Studies on Fluorescent Approach for Detecting Genetic Exchange in *Giardia duodenalis*

By

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Declaration

I declare that this thesis is my own account of research and does not contain any work that has been previously submitted for a degree at any tertiary educational institution.

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Abstract

The demonstration of a clonal population structure in epidemiological studies of *Giardia duodenalis* has been considered to support asexual reproduction in their life cycle. However, the exhibition of remarkable phenotypic and genetic heterogeneity within zoonotic assemblages of *G. duodenalis* (assemblages A and B) suggests the possible existence of sexual reproduction in the *Giardia* life cycle. To identify such a possibility, the aim of this study was to develop a modified transient and stable fluorescent expression systems to examine the possibility of sexual exchange between *G. duodenalis* isolates.

A commercial mammalian fluorescent plasmid was modified by cloning the glutamate dehydrogenase promoter upstream of the red fluorescent protein (pRFP). The plasmid was then transfected into the trophozoites of assemblages A and B by electroporation. Transient fluorescent trophozoites were observed in the assemblage B isolate but not in the assemblage A isolate. Furthermore, it was observed that the untransfected trophozoites outcompeted the transfected trophozoites, which hindered the selection process.

Therefore, to select the transfectants, a stable mammalian fluorescent vector, pRFPneo, and green fluorescent plasmid, pGFPneo, were constructed. The plasmid vectors were transfected into the assemblage A isolate separately. The transfected trophozoites were selected using drug G418. However, no stable cell line could be developed as all the transfected trophozoites were dead by day 4. The failure of stable transfection needs to be further investigated, particularly in vector construct.

As a conclusion, this study has demonstrated a transient expression in assemblage B was possible. The absence of fluorescent trophozoites in assemblage A suggested that both assemblages A and B require different transfection condition to express the exogenous gene. The improvements of transient and stable fluorescent system in this not only provide a platform to examine the possibility of sexual reproduction in future, but also assist in understanding the transcriptional activity of *Giardia*.

Acknowledgments

Unbelievable...my “honours” journey is at the end. Throughout this journey, I had never felt alone as I had so many “seniors” and friends who guided and supported me. Their kindness made my stressful and challenging “honours” journey enjoyable!

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Table of Contents

Chapter 2: Materials and Methods

Chapter 3: Results

Chapter 4:Discussions

List of Figures

List of Tables

Abbreviations

˚C	degree celcious
µg	micrograms
µl	microlitres
bp	base pairs
DNA	deoxyribonucleic acid
dNTP	deoxy-Nucleotide Triphosphate
EDTA	ethylenediamine tetra-acetic acid
g	gram
g	gravitational force
gdh	glutamate dehydrogenase
GFP	gree fluorescent protein
Kbp	kilo base pair
L	litre
LB	Luria Bertani
M	molar
min	minutes
mM	milimolar
ng	nanograms
PBS	Phosphate Buffer Saline
PCR	polymerase chain reaction
PKA	Protein Kinase A
pH	power of hydrogen
pmol	picamole
RFP	red fluorescent protein
rpm	rotations per minute

sec	seconds
spp	species
TAE	tris-acetate EDTA buffer
TE	tris-EDTA
UTR	untranslated region
v	volts
α	alpha
β	beta
Ω	ohms

Chapter 1

Introduction

1 Introduction

1.1 General Introduction

The flagellated protozoan *Giardia duodenalis* belongs to the Phylum Metamonada, class Trepomonadea, order Diplomonadida, and family Hexamitidae (Adam 1991; Thompson and Monis 2004). It is a major cause of enteric parasitic disease with approximately 2.8 x 10^8 cases of human giardiasis reported worldwide annually (Thompson 2004). The human isolates of *G. duodenalis* exhibit remarkable behavioural and genetic heterogeneity with its isolates, varying in mean generation time, *in vitro* and *in vivo* growth rates and drug sensitivities (Thompson and Monis 2004). It is not known whether such genetic variation is predisposed by a possible undiscovered mode of sexual reproduction or other genetic exchange factors (Thompson and Meloni 1993). The uncertainty about the mode of reproduction in the *G. duodenalis* life cycle is the major hindrance in understanding the cause of genetic variation. Hence, a fluorescent-based approach will be developed to detect the possibility of sexual exchange in the *Giardia* life cycle.

1.2 Introduction to *Giardia* spp

The genus "*Giardia*" was used for the first time in the 1880s when Kunstler named an organism that had been observed in tadpoles. Since then "*Giardia*" has been widely used to group this organism at the genus level (Adam 1991; Thompson and Monis 2004; van Keulen 2002). However, taxonomic determination at the species level has been perplexing and most challenging. This is because most designations at the species level are based on the host of origin and morphological differences observed using light microscopy, thus resulting in over 50 species names being proposed (Thompson 2004).

To avoid further confusion and overestimation of the number of species, Filice divided *Giardia* into three distinct species: *G. agilis*, *G. muris* and *G. duodenalis* (Figure 1.1). This is in accordance with morphological differences in *Giardia*'s median body, body shape and length (Adam 1991; Thompson and Monis 2004; van Keulen 2002). The scheme is widely accepted and constitutes the basis of the current *Giardia* taxonomy (Thompson and Monis 2004).

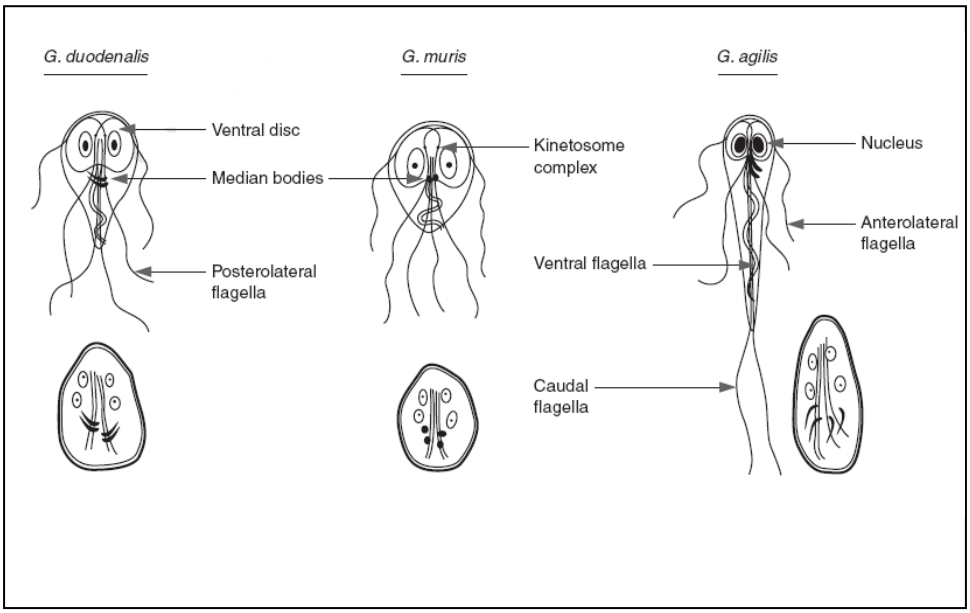

Figure 1.1: The morphology of trophozoite and cyst of *G. duodenalis*, *G. muris* and *G. agilis* (adapted from Thompson and Monis 2004)

1.3 Introduction to *Giardia duodenalis*

The scheme devised by Filice fails to reflect the considerable phenotypic and genetic heterogeneity that exists within *G. duodenalis*. The use of various genetic methods such as PCR, allozyme analysis, sequencing, RFLP analysis, phylogenetic analyses of nucleotide sequence data obtained for protein-encoding and rRNA genes had demonstrated that *G. duodenalis* is a complex species comprised of at least seven major assemblages with an identical morphology (Thompson and Monis 2004) (Table 1.1). However, at this stage, the development of proper species-level taxonomy is hampered by uncertainty about the sexuality and host specificity of the organism which has made species delineation based on host range untenable (Monis et al. 2003).

Genotype /Assemblage	**Host Range**
Zoonotic /A	Human, livestock ,cats dogs, beavers, guinea pig, slow loris
Zoonotic / B	Human, slow loris, chinchillas, dogs, beavers, rats, siamang
Dog / C & D	Dogs
Livestock / E	Cattles, sheep, pigs
Cat / F	Cats
Rat / G	Domestic rats
Muskrats/ Vole	Wild rodents

Table 1.1: *G. duodenalis* genotypes/assemblages (adapted from Thompson 2004

1.3.1 Epidemiology

G. duodenalis is globally distributed and can be transmitted via different ways such as waterborne, foodborne and direct faecal-oral routes (Hunter and Thompson 2005). *Giardia* infections are more frequently found in developing nations compared to developed nations, which may be due to poorer environmental hygiene, a higher degree of human to human contact and the hosts' low nutritional status (Flanagan 1992).

From a zoonotic standpoint, both assemblages A and B are thought to possess a higher zoonotic risk in comparison to other assemblages (Thompson 2000, 2004). This is because both assemblages exhibit low host specificity, infecting both human and animal hosts, particularly assemblage A-I isolates, which are more geographically widespread and infect most animals (Monis and Thompson 2003). Thus, they command the most attention with regards to having zoonotic potential (Thompson 2004).

To justify the zoonotic risk of *G. duodenalis*, more direct zoonotic transmission evidence is required. This is because current epidemiology genotyping and some cross-transmission studies are still insufficient to

conclude that the zoonotic transmission pathway is a primary pathway of human infection (Monis and Thompson 2003). In addition, most of the assemblage A and B isolates that have been identified in animals are not genetically identical to those found in humans (Thompson 2000).

1.3.2 Clinical Symptoms and Pathogenesis

Giardia is not invasive in either human or animal hosts, but its clinical manifestations can be divided into asymptomatic, acute and chronic infection (Farthing 1997; Homan and Mank 2001) (Figure 1.2). The exact pathogenesis of *G. duodenalis* infection is still poorly understood, however its pathophysiological role appears to involve villous atrophy and damage to the microvilli (Farthing 1997; Thompson 2000). Symptoms include acute or chronic diarrhoea, abdominal cramps and bloating, nausea, weight loss, dehydration and decreased appetite (Adam 1991; Thompson and Monis 2004).

The symptomatology associated with infection varies amongst individuals, although the reasons for this clinical heterogeneity have yet to be defined (Carnaby et al. 1994). It is suspected that the degree of infection can be influenced by hosts' nutritional, immune status, age and infection records

such as the presence of concurrent enteric disease (Flanagan 1992; Thompson and Monis 2004). In addition, the characteristics of the parasite may also have a role for determining the outcome of infection. As shown in a study by Aggarwal and Nash (1987), infection of two antigencally distinct isolates of *G. duodenalis* in the same host can induce different immune responses and required significantly different time to clear the infections. A few reports have also suggested that human giardiasis consists of mixed infections by phenotypically and genotypically different strains or subgroups resulting in the failure of drug treatment (Andrews et al. 1989; Carnaby et al. 1994; Mayrhofer et al. 1992).

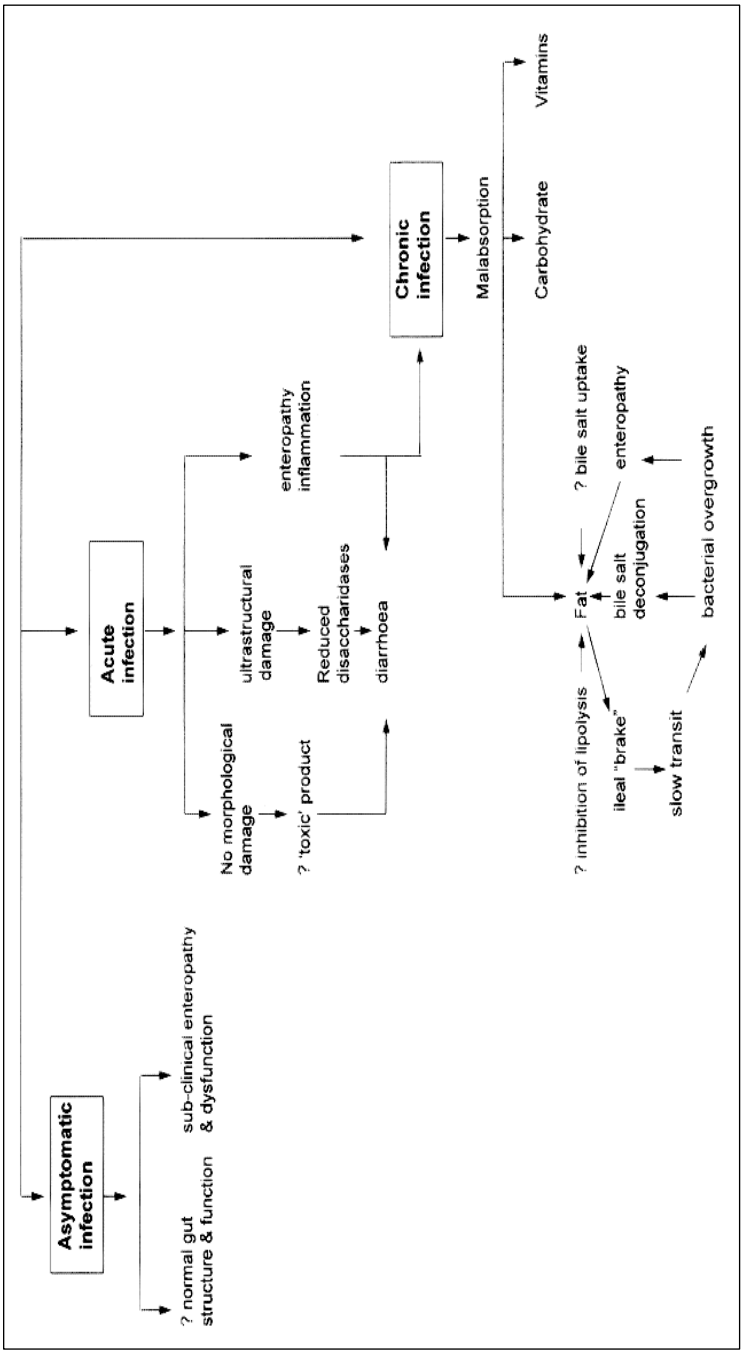

Figure 1.2: A model of *Giardia* pathogenesis (adapted from Farthing 1997)

1.4 The Cell Biology of *Giardia duodenalis*

1.4.1 Trophozoites

Trophozoites are the vegetative form of *G. duodenalis* (Figure 1.3). They are approximately 10-12 μm long and 5-7 μm wide, and are teardrop shaped with two nuclei of equal size at the anterior end (Lane and Lloyd 2002; Adam 2001). Trophozoites are bilaterally symmetrical, amitochondriate and have two median bodies that resemble claw hammers (Adam 2001). The shape of the median bodies is used to distinguish between different *Giardia* spp and is functionally related to the ventral disk (Adam 1991, 2001). The ventral disk is located on the anterior two thirds of the parasites ventral surface and is used for attachment to the intestinal mucosa (Adam 1991). *Giardia* also has four pairs of flagelle which includes an anterior pair, two posterior pairs and a caudal pair (Lane and Lloyd 2002). In contrast to the ventral disk, flagelle are more important for motility and the encystation process (Adam 2001).

Although *G. duodenalis* is in the order Diplomonadida, a Golgi-like apparatus has been reported in the encysting trophozoites (Lanfredi-

Rangel et al. 1999). Furthermore, *G. duodenalis* has always been described as an "anucleolated" organism but a recent study has demonstrated the presence of nucleoli (Jimènez-Garcìa et al 2008). Other distinct organelles including nuclei, lysosomal vacuoles, glycogen and ribosomal granules have been identified in trophozoites. However, peroxisome and smooth endoplasmic reticulum have yet to be reported (Adam 1991, 2001).

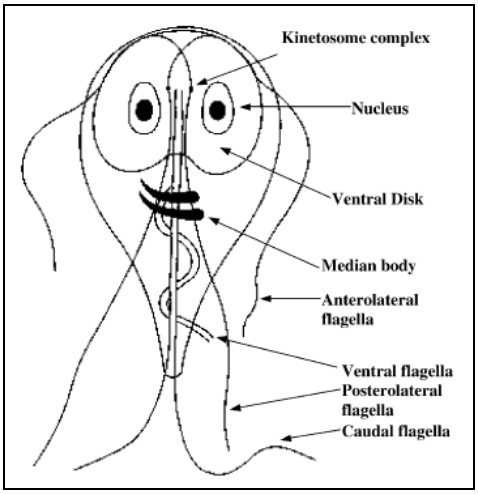

Figure 1.3: Morphological factures of *G. duodenalis* trophozoite (adapted from Monis and Thompson 2003)

1.4.2 Cyst Structure

Cysts are the infective form of *G. duodenalis* and ingestion of ten cysts is sufficient to cause infection (Cacciò and Ryan 2008). The cysts favour

moist and cool conditions from 4 ˚C to 10 ˚C in which they can remain viable for several months (Adam 2001; Flanagan 1992). Cysts are surrounded by a 0.3 μm to 0.5 um thick cell wall composed of an outer filamentous layer and an inner membranous layer separated by a thin layer of cytoplasm (Figure 1.4) (Adam 1991). Therefore, cysts are highly resistant to insult from the external environment such as chlorination, ozonolysis and gastric acid in the stomach of the infected host (Flanagan 1992).

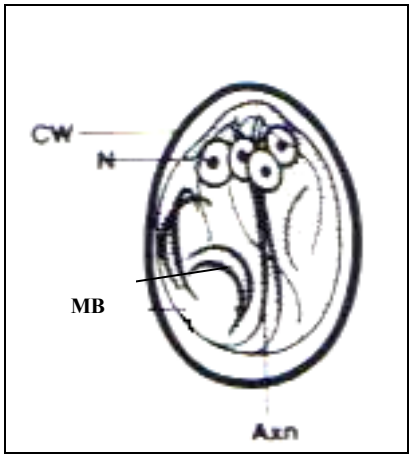

Figure 1.4: Morphological factures of *G. duodenalis* cyst (adapted from Meyer 1994)
CW=cell wall: Axn=axonemes: N=nucleus: MB= median body

1.4.3 Life Cycle

Giardia has a direct life cycle that involves only one host (Figure 1.5) (Lane and Lloyd 2002; Monis and Thompson 2003). Infection is initiated by the accidental ingestion of *Giardia* cysts and then follow by the colonisation (excystation, adherence and multiplication) of the small intestine (Gillin, Reiner, and McCaffery 1996; Lane and Lloyd 2002; Thompson 2000; Flanagan 1992). While passing through the small intestine and large intestine, it undergoes encystation and the cysts are excreted to the environment in faeces (Monis and Thompson 2003).

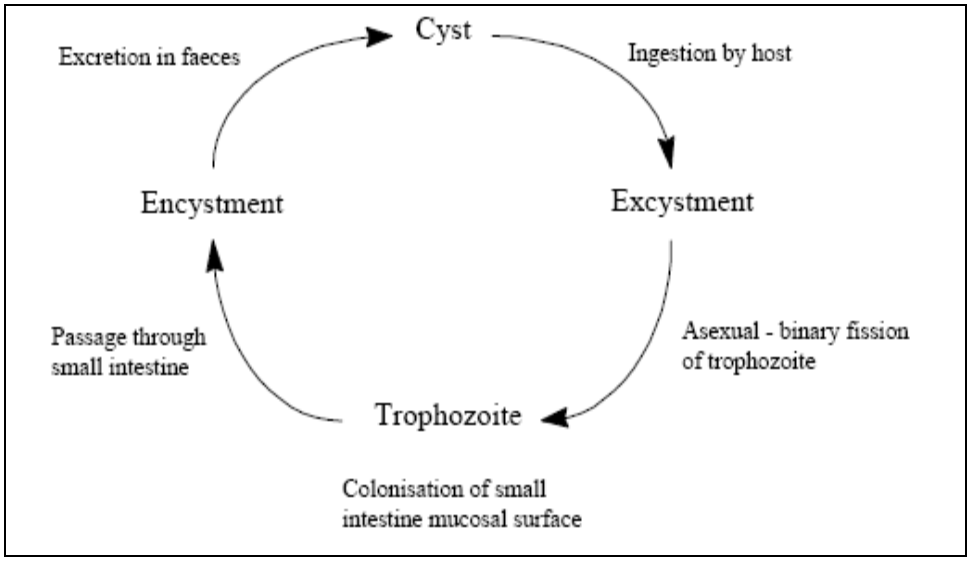

Figure 1.5: The life cycle of *G. duodenalis* (Monis and Thompson 2003)

1.4.3.1 Excystation

The excystation process is fast and can be completed in 10 minutes (Erlandsen, Weissner, and Ottenwaelter 2002). Excystation may be induced by a series of signal transductions, such as calmodulin and PKA which stimulate dephosphorylation of protein in the intracellular vacuoles and the discharge of their contents (Coggins and Schaefer 1986). The discharged contents include substances such as lysosomal acid phosphatase, which will dephosphorylate protein to initiate the first step of excystation (Coggins and Schaefer 1986; Farthing 1997; Svard, Hagblom, and Palm 2003). Subsequently, the release of the newly excysted cell, excyzoite, by cysteine protease activity is the first sign of excyzotic activity (Ward et al. 1997; Bernander, Palm, and Svard 2001).

During excystation (Figure 1.6), the excyzoite generates 4 trophozoites. The quadrinucleate cell with 16 N (4 x 4N) undergoes first cytokinesis to form two cells with two 8N nuclei (2x4N) (Bernander, Palm, and Svard 2001; Svard, Hagblom, and Palm 2003). The cell then divides into two excyzoites with two nuclei and a cellular ploidy of 4N via nuclear division. A further process of cytokinesis gives rise to two trophozoites with two diploid nuclei each (Svard, Hagblom, and Palm 2003). Therefore, a single

cyst produces four trophozoites (Bernander, Palm, and Svard 2001; Svard, Hagblom, and Palm 2003)

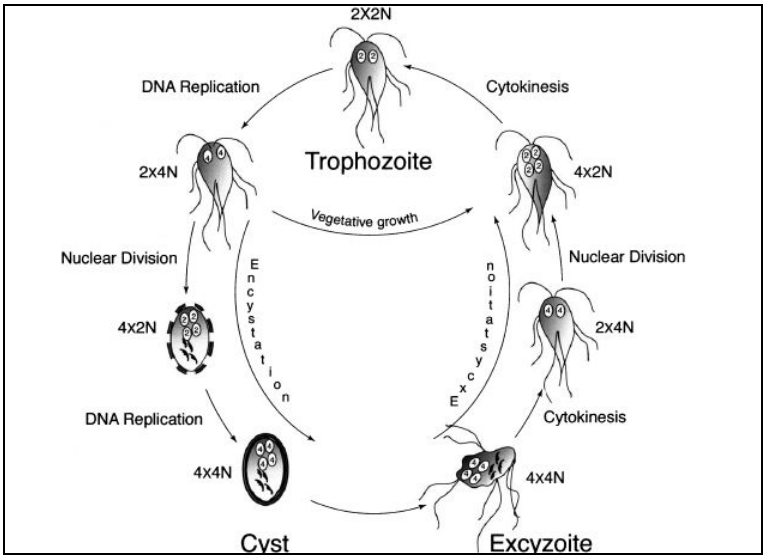

Figure 1.6: *Giardia* vegetative life cycle (adapted from Svard, Hagblom, and Palm 2003)

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After excystation, trophozoites attach intimately to the intestinal epithelium using the ventral disk with the aid of the flagella (Farthing 1997). The attachment of trophozoites is followed by their multiplication (Farthing 1997) with the number of trophozoites increasing exponentially by binary fission (Yu, Birky, and Adam 2002). However, during cell division , the trophozoite is non-adherent and motile (Benchimol 2004).

1.4.3.2 Encystation

Trophozoites gradually move to the lower part of the small intestine propelled by the flagella, median body and funis (Ghosh et al. 2001). The movement is controlled by the caudal flagella and their beating synchronises the movement of three other pairs of flagella (Ghosh et al. 2001). When the trophozoite migrates from the acidic stomach environment to the near neutral duodenal and jejunal pH, encystation is initiated (Hausen, Freitas Jr, and Monteiro-Leal 2006).

Encystation is divided into early and late phases. In early encystation, the trophozoite multiplication process is halted, they then change into a round shape that is no longer able to attach to the surfaces (Svard, Hagblom, and Palm 2003). The specific encystation antigens are expressed with the high molecular weight antigens for cyst wall construction and the down-regulation of trophozoite-specific genes (Adam, 1991, Erlandsen et al., 1990, Svard et al., 2003). Golgi-like structures also become visible at this time (Gillin et al. 1987). During the late encystation phase, the assembly of cyst wall filaments is initiated at the trophozoite plasmalemma sites (Adam 2001) and both nuclei will then undergo two rounds of DNA

replication, yielding a cyst with four nuclei and 16 N (Figure 1.6) (Bernander et al., 2001, Svard et al., 2003).

1.5 Molecular Biology of *Giardia duodenalis*

G. duodenalis is a diploid organism that has two nuclei that replicate at approximately the same time and both are transcriptionally active (Kabnick and Peattie 1990). Studies have shown that each nuclei may contain a full complement of DNA as approximately equal copies of rRNA genes were found in each nucleus (Adam, Nash, and Wellems 1991; Kabnick and Peattie 1990). However, a recent cytogenetic study proposed that *Giardia* nuclei are not equivalent, as each nucleus possesses different number and size of chromosomes.

G. duodenalis has a small compact genome, ranging in size from 3.0 $x10^7$ (Nash, McCutchan, and Keister 1985) to 8.0 $x10^7$bp (Boothroyd et al. 1987). It has five distinct chromosomes ranging in size from 1.6Mb to 3.8Mb (Adam 2000) which are all closely related and replicate almost simultaneously (Adam, Nash, and Wellems 1988; Tumova et al. 2007). The constant rearrangement and recombination of rDNA (Le Blancq and Adam 1998), rRNA (Hou et al. 1995) and variant-specific surface protein (vsp) genes (Adam et al. 1988) have contributed to the diversity of *G. duodenalis* chromosomes (Adam 2000). In addition, some of the

duplicated chromosomes appear to have the same size range and have now been recognized as minor bands that represent "homologous" chromosomes that are responsible for the heterogeneity of *Giardia*'s karyotypes (Adam 2000). Therefore, *G. duodenalis* is also suspected to be a polyploid organism.

The *G. duodenalis* genome has around 42 % to 48 % GC nucleotides and also contains GC rich rRNA (Adam 2000). *G. duodenalis* rRNA appears to be much smaller than eukaryotes and eubacteria, and is comprised of 28S large subunits and 16S small subunits (Adam 1991). The study of *G. duodenalis* transcription and translation has also revealed a 5' untranslated region (UTR) that is only 6 nucleotides in length and no RNA capping has been found (Kirk-Mason et al., 1989; Yu et al., 1998). A short *G. duodenalis* 3'UTR, putative polyadenylation signal and a potential TATAA box have also been reported (Adam, 2001, Gillin et al., 1990). A Shine-Dalgarno sequence was identified within 5 to 10 nucleotides downstream of the coding region (Yu et al. 1998). Like other eukaryotic cells, the linear chromosomes are flanked by telomeres that form a nucleosome when associated with four core histones (H2a, H2b, H3 and h4) and a linker histone (H1) (Adam, 2000, Wu et al., 2000, Yee et al., 2007).

1.6 Mode of Reproduction

1.6.1 Clonality

It is not known whether the genetic variation in *G. duodenalis* is the consequence of undiscovered sexual exchange. Up till now, most of the molecular epidemiological studies have been largely consistent with an asexual mode of reproduction (Andrews et al. 1989; Andrews et al. 1992; Meloni, Lymbery, and Thompson 1988; Meloni, Lymbery, and Thompson 1989, 1995; Cooper et al. 2007). Asexual reproduction is a process whereby the daughter cells are reproduced from a single parent organism by mitotic division. The daughter cells are genetically identical to parental cells as mitosis only involves DNA replication and segregation of identical chromatids into two daughter cells (Becker, Kleinsmith, and Hardin 2003)

The clonality of *G. duodenalis* was supported by the correlation of independent sets of genetic markers and widely identical zymodemes (M1) that have been found in Australia, Mexico, New Guinea and Poland (Figure 1.7) (Meloni, Lymbery, and Thompson 1988; Thompson and Meloni 1993; Meloni, Lymbery, and Thompson 1995). This indicates *G.*

duodenalis is an asexually reproducing organism since the genotype is replicated as a unit and persists over time and space. The wide distribution of genotypes may be due to several factors such as the lack of recombination, historical factors and possible selective differences amongst clones (Tibayrenc and Ayala 1987) .

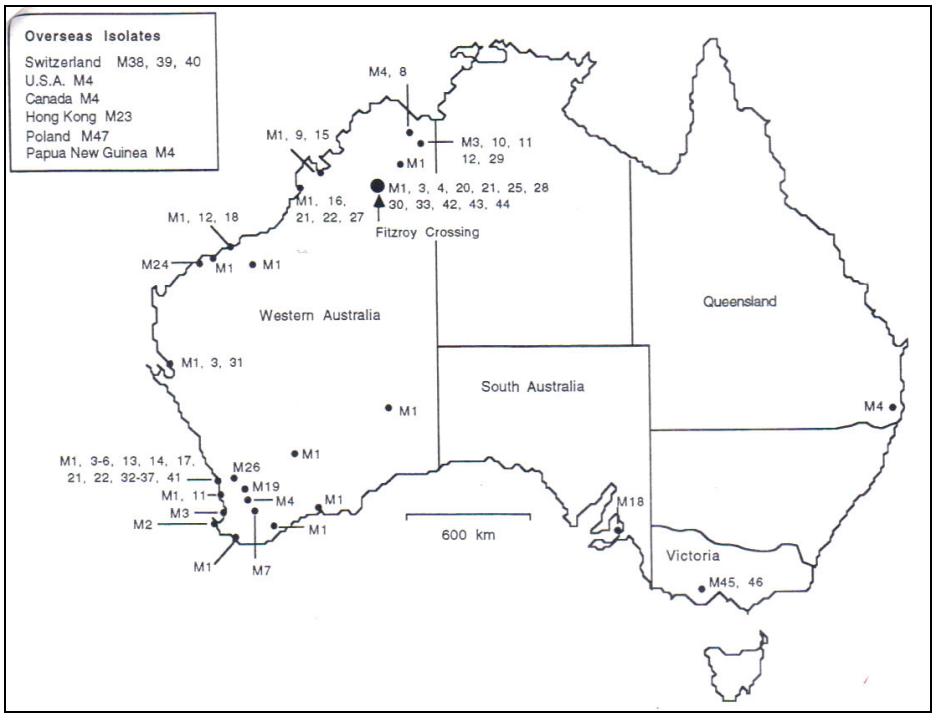


Figure 1.7: Geographical distribution of zymodemes of *G. duodenalis* (adapted from Meloni et al. 1995)

Further supporting the asexual reproduction of *Giardia*, Tumova et al. (2007) not only showed that *Giardia* nuclei are not equivalent but also proposed that the stable occurrence of aneuploid (odd chromosome

number) in one or both of nuclei is indicating a long-term asexual reproduction in *Giardia* life cycle. Furthermore, enzyme electrophorectic studies of *Giardia* had shown predominantly single banded patterns with different isolates often having different isoenzymes (Meloni, Lymbery, and Thompson 1995). This suggests that *G. duodenalis* is an asexual, functionally haploid organism, with varying enzymes that arise from occasional mutations (Meloni, Lymbery, and Thompson 1995, 1989).

However, G. *duodenalis* is a diploid or polyploid organism with asexual reproduction and the accumulation of mutation without recombination should produced substantial heterozygosity in each nucleus (Meloni, Lymbery, and Thompson 1995). Therefore, in the same study, some isolates produced multiple-banded enzyme patterns (Meloni, Lymbery, and Thompson 1995). The exact reason why some isolates produced a single-banded pattern was not known, but this may be due to a possibility of sexual reproduction in their life cycle (Thompson and Meloni 1993).

1.6.2 Evidence for Sexual Reproduction

Sexual reproduction is a process involving genetic exchange from two parents and producing genetically distinct daughter cells. Sexual reproduction not only involves mitosis but also meiotic processes to generate genetic diversity by crossing over and recombination of genes and passage to daughter cells (Becker et al. 2003).

Although it is evident that *Giardia* has a clonal population structure, this does not rule out the possibility of sexual recombination in their life cycle. Other protozoan parasites such as *Trypanosoma brucei* (MacLeod, Tait, and Turner 2001), and *Entamoeba histolytica* (Sargeaunt 1985) also have clonal genetic structures in nature, but have been shown to undergo sexual recombination in the laboratory.

1.6.2.1 Allelic Heterozygosity

The presence of uncommonly low allelic heterozygosity at the sequence level in some population genetic studies greatly supports the idea that there is potential for sexual reproduction occurring in *G. duodenalis*. In a genetic study, WB clone 6 (A-I) isolates demonstrated a low heterozygosity of less than 0.01% (Lasek-Nesselquist, personal

communication). The triose phosphate isomerase gene (*tim)* sequence from multiple isolates also revealed a low allelic heterozygosity of around 0.02% (Baruch, Isaac-Renton, and Adam 1996). This would be unlikely if *G. duodenalis* undergoes purely asexual reproduction. Asexual organisms do not possess DNA repairing systems thus should accumulate substantial mutatated gene (Meloni, Lymbery, and Thompson 1995). Furthermore, the homologous chromosomes within each nucleus will differentiate independently (mitosis) from one to another via the accumulation of rearrangement and nondisjunction process during evolution (Tumova et al. 2007). Therefore, *Giardia* should have a substantial heterozygosity at most loci (Meloni, Lymbery, and Thompson 1995). The exhibition of such low degrees of allelic heterozygosity (0.05% to 0.1%) is more likely to occur in sexual organisms (Yu, Birky, and Adam 2002).

1.6.2.2 Cell Differentiation

The cell differentiation in *G. duodenalis* is proposed to be a remnant form of meiosis as the excysted cell divides twice (cytokinesis) without DNA replication (Bernander, Palm, and Svard 2001; Svard, Hagblom, and Palm 2003). The division of trophozoites by cytokinesis can also be

demonstrated by three models of division which include ventral-dorsal, ventral-ventral and dorsal-dorsal divisions (Figure 1.8) (Benchimol 2004).

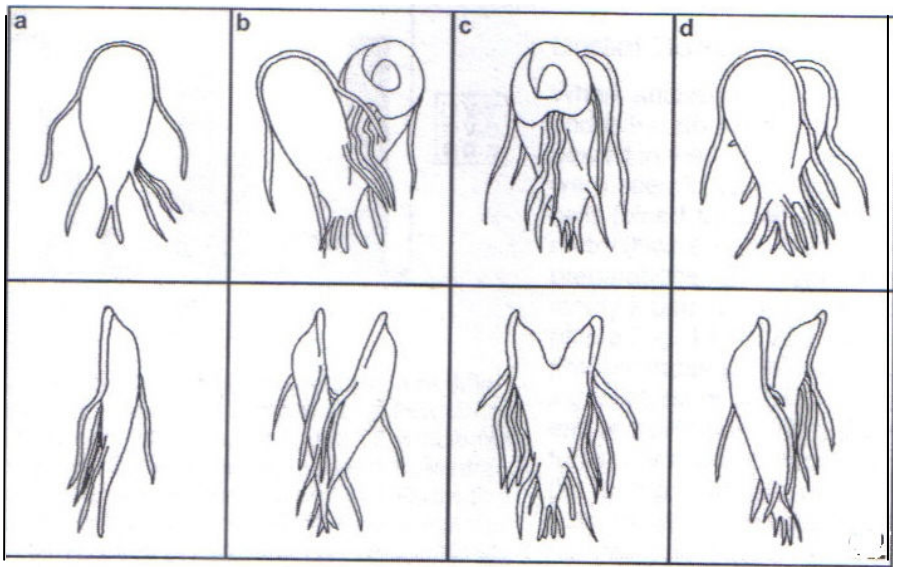


Figure 1.8: The possible mode of cytokinesis (adapted from Benchimol 2004)
First row= dorsal view Second row= lateral view
a) interphasic cell
b) ventral-ventral division
c) dorsal-dorsal division
d) ventral-dorsal division

Benchimol (2004) has suggested that dorsal-dorsal cytokinesis is more likely due to sexual reproduction since it evokes fusion rather than a gradual fission between daughter and parent cell. The presence of different modes of cytokinesis also suggests the existence of a meiotic process in which the cells will divide in one mode during the first division and in

another cytokinetic mode in the second meiosis division (Benchimol 2004).

Furthermore, it is most likely that the *Giardia* encystation process is an ancestral form of sexual reproduction (Bernander, Palm, and Svard 2001; Svard, Hagblom, and Palm 2003). This idea is further supported by a recent study where an episomal plasmid was transferred between two nuclei in a cyst indicating a genetic exchange between the nuclei (Poxleitner et al. 2008). In addition, the study also demonstrated that karyogamy process and somatic homologous recombination occurred under the influence of a meiosis gene homolog in the cysts but not in trophozoites (Poxleitner et al. 2008). This is because the two nuclei in the trophozoites remain physically and genetically distinct during mitosis (Sagolla et al. 2006).

1.6.2.3 Others

The meiosis-specific (Ramesh, Malik, and Logsdon 2005) and spermatogenesis genes (Bernander, Palm, and Svard 2001) that are widely present in sexual eukaryotes have been identified in *G. duodenalis*. Furthermore, the identification of meiotic paring and chromosome reassortment also suggest a possibility of sexual reproduction in *G.*

duodenalis (Cooper et al. 2007). The presence of a double-stranded RNA virus in *G. duodenalis* has also further supported the possibility of additional genetic recombination (Teodorovic et al., 2007; Wang et al., 1993). Moreover, reductional partitioning or non-disjunction of nuclei has also been detected in a study by Yu et al. (2002) who proposed that only sexual reproduction is able to maintain such a high homozygosity population. The sexual reproduction theory is further supported by the detection of antigenic variation or variant surface protein (vsp), which express only one antigen at a time, switching from one to another. The relocation of genes is unlikely to happen as vsp genes are not telomeric and recombination or relocation to other parts of the genome has not been documented (Adam 2000). A study by Lasek-Nessequist (personal communication) has shown the closest evidence of sexual reproduction for *G. duodenalis*, where the alleles of assemblage A were found within an assemblage B isolate (BAH12c4).

1.6.3 Implication

Based on the above evidence, the possibility of genetic exchange or sexual reproduction in the life cycle of *Giardia* cannot be ruled out although clonal propagation is still the predominant mode of reproduction. The

occurrence of sexual reproduction may be too rare to be detected in a predominant clonal population (Tibayrenc and Ayala 2002).

The identification of potential sexual exchange has significance in epidemiological studies such as the relationship between the selection pressure and degree of heterogeneity in a population (Green and Noakes 1995). The degree of heterogeneity within a *G. duodenalis* population may be affected by environmental conditions since the genetic heterogeneity within a population may vary from one endemic area to another. In an epidemiological study in North Western Australia, the genetic diversity of a human isolate of *G. duodenalis* was 10^2-10^3 times greater than populations from central Europe (Meloni et al. 1991). It implies that selection pressures from frequent drug exposure or other environment factors are acting on a genetic population of the parasite with a high degree of spontaneous heterogeneity (Thompson and Meloni 1993). This phenomenon was similar to *Plasmodium* studies where sexual reproduction was more likely to occur in the areas with high transmission rates (Gauthier and Tibayrenc 2005). This suggests that the occurrence of sexual reproduction may be a response of the parasite to the selective pressures.

1.7 Genetic Manipulation of *Giardia duodenalis*

1.7.1 *In vitro* Cultivation

Due to the extensive genetic diversity of *G. duodenalis*, only assemblages A, B and E have been successfully grown axenically. This is not surprising as each assemblage has its own host-preference and different metabolic and biochemical requirements, mean generation times and pH preferences (Thompson and Monis 2004). Axenisation of assemblages C and D all appeared to be refractory to culture (Thompson and Monis 2004).

1.7.2 Transfection Systems

Giardia transfection systems are a combination of both cellular and molecular techniques (Davis-Hayman and Nash 2002). The transfection system may be divided into transient transfection and stable transfection systems (Adam, 2001; Lewin, 2006). Transient transfection systems only allow the transfected gene to be retained for a short period of time as it is lost during mitosis due to unstable integration (Lewin 2006). Conversely, stable transfection can retain the transfectant under drug selection,

therefore retaining the inserted DNA beyond reproduction. These systems involve the use of electroporation of the nucleic acid or plasmid into the organism (Lewin 2006).

These two transfection systems are further divided into DNA-based transfection and viral-based transfection systems (Table 1.2). The first transient DNA-transfection system was reported in 1995 by Yee and Nash where luciferase flanked the 5' end of a short region of the glutamate dehydrogenase gene and possessed 3' end of a putative polyadenylation signal which allowed the formation of heterologous DNA (Yee and Nash, 1995). In addition, *Giardia* promoter sequences such as glutamate dehydrogenase gene (Yee et al., 2000), ran (Sun and Tai 1999), actin (Drouin, De Sa, and Zuker 1995), and α-tubulin (Elmendorf et al. 2001) have been used to control the expression of the reporter gene.

Before the introduction of DNA based transfecting systems, the genetic study of *G. duodenalis* was performed using *Giardia* virus transfection systems. *Giardia* virus is a nonsegmented, dsRNA virus of the *Totiviridae* family that infects only *G. duodenalis* (Yu et al. 1995). A single transcript of the dsRNA genome is isolated and modified to carry the gene of interest. It will then be transfected into the trophozoites by electroporation

to initiate infection and replication (Yu et al., 1996). The transfected trophozoites can then be selected with geneticin, neomycin and puromycin (Davis-Hayman and Nash 2002). This system is very consistent and persistent with strong expression of the reporter gene (Yu et al., 1995).

Table 1.2: The comparison of DNA based transfection and RNA virus-based transfection (Davis-Hayman and Nash 2002)

Parameter	DNA-based transfection	RNA virus-based transfection
DNA/RNA	Transient: 50.0 µg circular plasmid; stable: 10.0 µg circular or linearized plasmid	100 µg purified transcript; must add RNase inhibitors; in vitro transcribed from plasmid DNA
Giardia	10^7 cells/0.3 ml of media	4×10^6 in 0.8 ml cytomix buffer; previously infected with wild type GLV (MOI = 1000)
Selectable markers	Puromycin-*N*-acetyltransferase gene (*pac*)[a]; neomycin phosophotransferase gene (*neo*)[b]	Puromycin-*N*-acetyltransferase gene (*pac*); neomycin phosophotransferase gene (*neo*)
Number of genes[c]	3	2
Cuvette	0.4 cm	0.4 cm
Volts	350	1000/cm
µF	1000	500
Ohms	720	-
Pulse number	1	2
Time constant	-	6.4 ms

[a] Select with 100 µM puromycin.
[b] Select with 600 µg ml^{-1} geneticin.
[c] Highest number of genes expressed in one transfected cell line. For DNA-based transfection, the *luc*, *pac* and *Tet* repressor were expressed from one plasmid. For viral-based system, *luc* and *pac* were expressed from a bicistronic mRNA.

1.8 Aims and Hypotheses

The aim of this study was to examine the possibility of genetic exchange between *G. duodenalis* isolates using a modified mammalian fluorescent expression system. The sexual reproduction of *G. duodenalis* will be identified based on dominant selectable markers and fluorescent protein transgenes. Two populations of *G. duodenalis* will be transfected separately with red and green fluorescent protein and mixed. The co-expression of both red and green fluorescence will produce a yellow fluorescence that may indicate a potential sexual exchange in *G. duodenalis* (Figure 1.9).

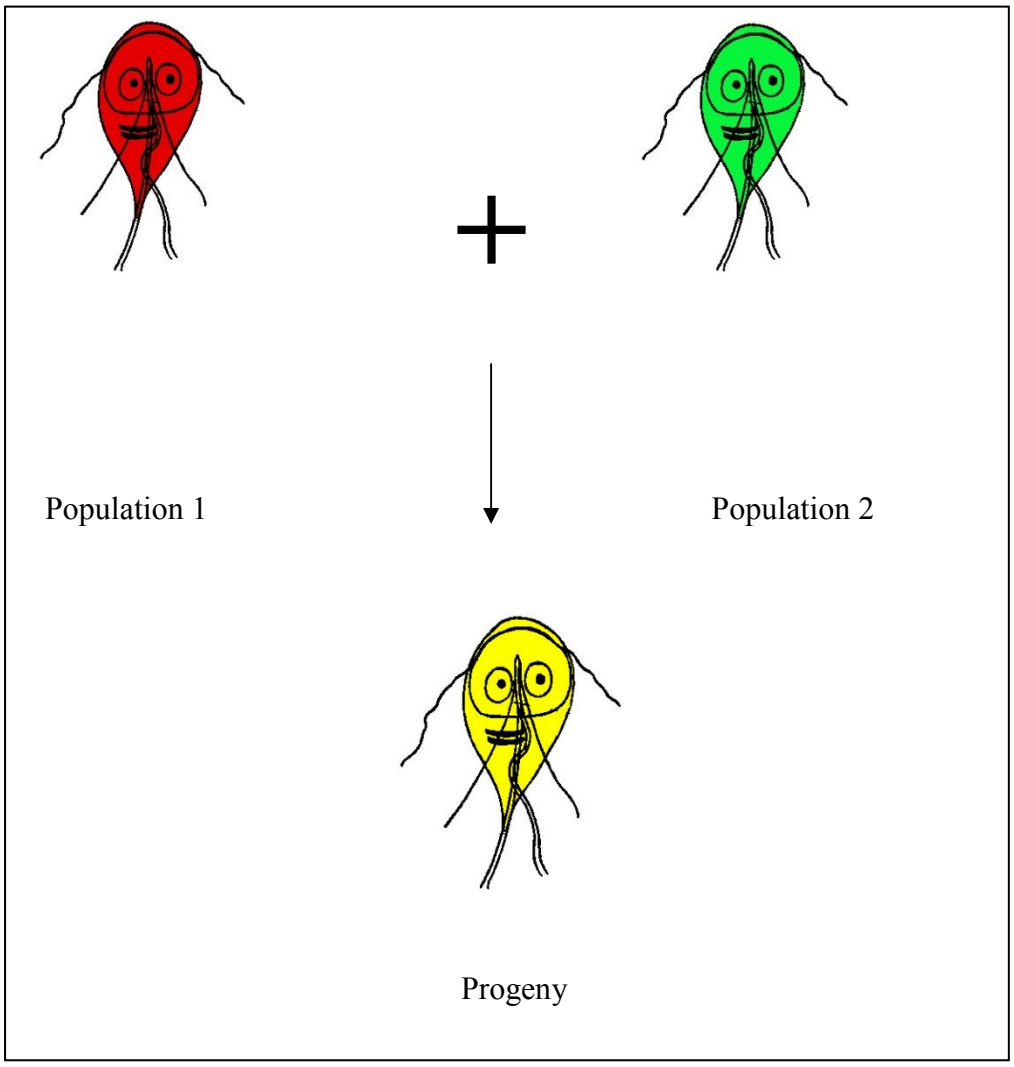


Figure 1.9: Diagram illustrating the design of the experimental cross of two populations

However, before performing the mixing population experiment, a novel mammalian fluorescent transfection construct will be developed to transfect trophozoites.

Chapter 1: Introduction

In this study, a 44 bp glutamate dehydrogenase promoter will be used to drive the expression of both red and green fluorescent proteins. This is because the mammalian promoter, SV40 (simian virus 40), is not functional in *Giardia* and the glutamate dehydrogenase gene is one of the most highly conserved genes in *Giardia*, and is expressed constitutively in both excystation and encystations stage (Yee and Nash 1995). The constructed vector will then be transfected transiently and stably into assemblage A and B isolates and their growth monitored microscopically.

Therefore, the hypotheses of this study are driven by the methodology:

1. Transient mammalian fluorescent expression is possible in transfected trophozoites .
2. The reporter gene can be induced by *Giardia* glutamate dehydrogenase minimal promoter.
3. Stable mammalian fluorescent expression is possible in transfected trophozoites.
4. Neomycin resistance expression can be induced by *Giardia* glutamate dehydrogenase minimal promoter.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 General

2.1.1 *Giardia duodenalis* Culture

The trophozoites of *Giardia duodenalis* assemblage A (BAH 3c3 and BAH 2c1) and assemblage B (BAH 34c8) isolates were used for this study. The axenic cultures were maintained anaerobically in BS-1-33 *Giardia* medium at 37˚C in 10 mL borosilicate tubes (Table 2.1). The isolates were subcultured every 48 hours by chilling the tube on ice for 15-30 minutes to detach the trophozoites from the flask surface. The tube was mixed by inversion to disperse the trophozoites and 1 mL was then transferred to a new culture flask containing new culture medium. Aseptic techniques were used at all times. The growth of trophozoites was monitored microscopically using an inverted microscope (model BXK 51, Olympus, Japan).

Table 2.1: BS-1-33 *G. duodenalis* culture media

BS-1-33 Giardia Culture	**30 g**	**Biosate Peptone**
	10 g	**Glucose**
	2 g	**NaCl**
	2 g	**Cysteine HCl**
	1 g	**K_2HPO_4**
	0.6 g	**KH_2PO_4**
	0.01 g	**Ferric Ammonia Acid**
	0.2 g	**Ascorbic Acid**
	0.5 g	**Bile**

The pH was adjusted to 7-7.2 with 1 M NaOH before the addition of 100 mL of heat inactivated serum. Made up to 1 L with water and filter sterilised into a sterile 1 L bottle.

2.1.2 Agarose Gel Electrophoresis

DNA products were electrophoresed at 80 V for 100 minutes in a 0.5 µg/mL solution of 1 x 10,000 Sybr®Safe in DMSO (Invitrogen, Carlsbad, CA) stained 2.5 % agarose gel. The buffer for agarose gel electrophoresis was 1 x Tris.Acetate.EDTA (TAE). The agarose gel was electrophoresed on Bio-Rad Mini Sub™ tank (Bio-Rad, Richmond, CA, USA). DNA markers of known fragment sizes (2kbp ladder) (Fisher Biotec, Australia) were used to estimate the sizes of DNA. The gel was visualised under visible light transilluminator at a wavelength of 520 nm (Fisher Biotec).

2.1.3 Agarose Gel Purification

The desired DNA fragment was extracted and purified according to the "PureLink™ Quick Gel Extraction Kit" (Invitrogen). Briefly, six volumes (1 mg = 1 mL) of Gel Purification Buffer (GS1) was added to the 2.5 % agarose gel slice. The gel slice together with appropriate volumes of Gel Purification Buffer was incubated for 15 minutes at 50 ˚C until the gel dissolved. The solution was then transferred to a Quick gel extraction column, and centrifuged for 1 minute at 13,000 x *g* (Eppendorf Centrifuge 5417C, Northbrook, IL). The flow-through solution was discarded, and the sample was washed with 500 µL of Gel Purification Buffer. The sample was then incubated at room temperature for 5 minutes, and centrifuged at 13,000 x *g* for one minute. The flow-through was discarded, 700 µL of Wash Buffer (W9) was added to the column and the sample was centrifuged at 13,000 x *g* for one minute. The flow-through was discarded and the sample was further centrifuged for one minute. The DNA was then eluted into a fresh tube using 20 µL of Buffer EB and stored at -20 ˚C. The DNA concentration was quantified as described in the NanoDrop®ND1000 manual (NanoDrop Technologies, Inc, USA).

2.1.4 Ligation

Each ligation reaction contained 100 ng of plasmid vector, 1 x ligation buffer, three units of T4 DNA Ligase (Promega, WI, USA), and the appropriate amount of inserted DNA to give a 1:3 and 1: 8 ratio of vector: insert in a final volume of 10 μL. The reaction tube was incubated overnight at 4 ˚C.

2.1.5 Transformation

The ligation product was transformed into competent JM109 *Escherichia coli* (Promega) using the following method:

1. The competent cells were thawed on ice and 50 μL of cells were transferred into a 1.5 mL microcentrifuge tube on ice.

2. Five microlitres of ligation product was added to the cell mixture and mixed gently by tapping the tube.

3. The mixture was placed on ice for 30 minutes, then heat shocked at 37 ˚C for 5 minutes and placed on ice for a further 5 minutes.

4. Nine hundred and fifty microlitres of room temperature Luria Bertani (LB) broth was added into the tube and incubated at 37 ˚C for 60 minutes.

5. To obtain higher number of colonies per plate, the cells were pelleted by centrifugation at 10, 000 x *g* for 15 seconds, resuspended with 200 µL of LB broth, and 100 µL was plated onto an LB plate which contained 50 mg/mL of kanamycin (Sigma, Missouri, USA). The plates were incubated overnight at 37 ˚C in an inverted position.

2.1.6 Selection and Growth of White Colonies

The white colonies containing the insert were picked from the LB plate and inoculated into individual McCartney bottles, each containing 10 mL LB broth and 50 mg/mL of kanamycin. The culture tubes were then incubated overnight at 37 ˚C on a gyratory shaker at 225 rpm.

2.1.7 Plasmid Preparation

Plasmid DNA from overnight *E. coli* cultures were purified according to the QIAprep® Miniprep protocol, catalogue number 27104 (Qiagen, USA). Briefly, the overnight cultures were transferred into a 15 mL sterile centrifuge tube and centrifuged at maximum speed for 15 minutes to pellet the cells. The pelleted cells were resuspended in 250 µL of Buffer P1 and then transferred to 1.5 mL microcentrifuge tubes. This was followed by the addition of 250 µL of Buffer P2 and mixed thoroughly by inverting the tube several times. Three hundred and fifty microlitres of Buffer N3 was then added, mixed immediately and centrifuged at 17,900 x *g* for 10 minutes. The supernatant was decanted into QIAprep™ spin column and centrifuged at 17, 900 x *g* for 10 minutes, the flow-through was discarded. The spin column was washed by adding 0.5 mL Buffer PB and centrifuging for 60 seconds, after which the flow-through was discarded. This was followed by the addition of 0.5 mL Buffer PB and centrifuging for 30-60 seconds. The flow-through was discarded and the tube was centrifuged for one minute at 17,900 x *g* to remove the residual wash buffer. The QIAprep™ column was then placed in a 1.5 mL microcentrifuge tube, and 50 µL of Buffer EB was used to elute the DNA.

The eluted DNA was quantified with NanoDrop®ND1000 spectrophotometer.

2.1.8 DNA Sequencing

The Big Dye™ terminator method was used to perform DNA sequencing. For each separate reaction, the plasmid was sequenced using plasmid forward primer and inserted DNA reverse primer. Each reaction mixture contained 2 µL of Big dye terminator mix (Applied Biosystems, Foster City, California), 1 µL of 5 x sequencing buffer, 3.2 pmole of each sequencing primer, and the required amount of plasmid DNA/ PCR product. This was made up to a total volume of 10 µL with distilled water.

Thermal cycling conditions for the sequencing reaction are as shown in figure 2.1.

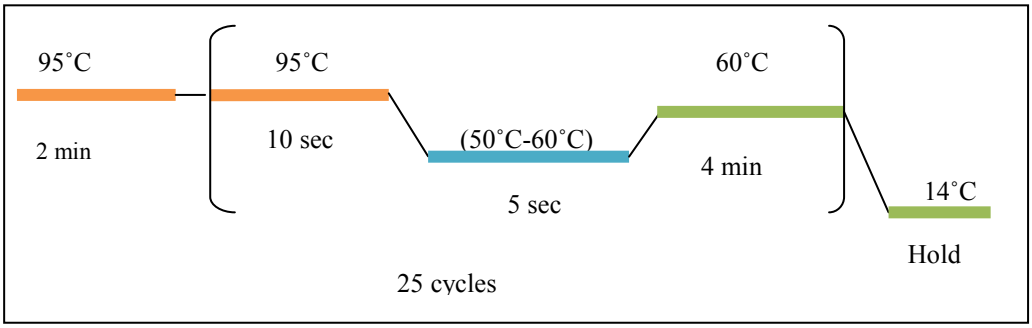


Figure 2.1: Thermal cycling conditions for sequencing reaction

Following the thermal cycling reaction, the product was ethanol precipitated by adding 1 µL of 3 M sodium acetate and 25 µL of 100 % ethanol and mixed. The tubes were incubated at room temperature for 20 minutes, after which they were centrifuged at maximum speed for 30 minutes, and the supernatant was removed. The pelleted DNA was rinsed with 125 µL of 70% ethanol and centrifuged at maximum speed for 5 minutes, the supernatant was removed and the sample air-dried on the bench in the dark for 15 minutes.

2.1.9 Sequencing Analysis

The sequencing results were edited using Finch TV (http://www.geospiza.com/finchtv/.html) and Gene Tool was used to align the sequences (http://wwww.biotools.com/download/productinfo.html).

2.2 Transient Plasmid pRFP Vector Construct

2.2.1 Synthetic Glutamate Dehydrogenase Promoter Construct

The commercial promoterless plasmid containing the red fluorescent protein, pTurboRFP (Evrogen, Russia) was used as the basis for the synthesis of all constructs (Figure 2.2).

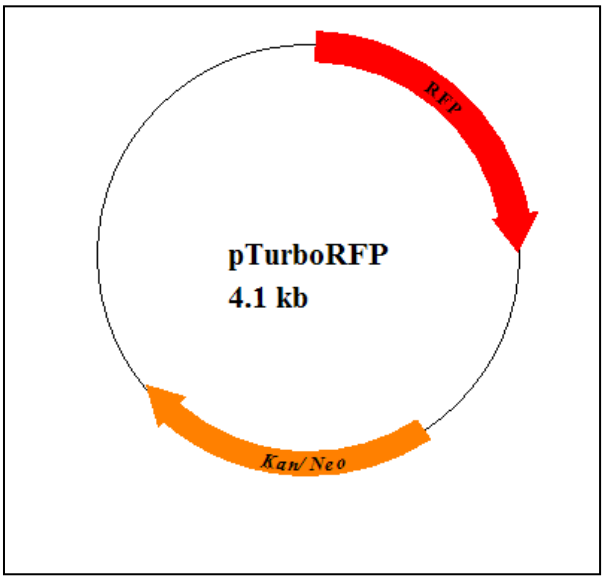


Figure 2.2: Promoterless pTurboRFP vector

Therefore, in order to drive the expression of red fluorescent protein in *G. duodenalis*, a synthetic glutamate dehydrogenase minimal promoter as described by Yee et al. (2000) was inserted upstream of the red fluorescent protein sequences and referred to as the pRFP plasmid (Figure 2.3).

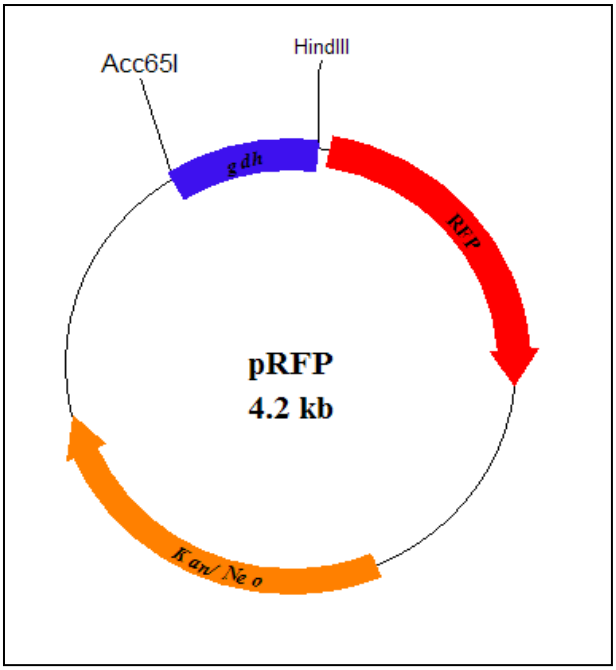


Figure 2.3: Transient plasmid pRFP vector

The sequences of *Giardia duodenalis* glutamate dehydrogenase (accession number M84604) were obtained from the NCBI nucleotide database search (http://www.ncbi.nlm.nih.gov/). According to Janet et al. (2000), the coding sequence of the 44 bp minimal promoter located immediately upstream of the translational start sites is located from base 180 to base 223 and is shown as below. The relevant sequence motifs include ATG

starting codon (underlined), the upstream AT-rich elements region (highlighted), the transcription initiation sites (dark bold fonts) and CAAT element (purple fonts).

5'-GACCACAAATAACGCCTTTAATTACAGGCGCCCCAGATTTTTAAAATG-3'

To integrate the synthetic minimal promoter into the commercial promoterless plasmid, the unique restriction site *Acc65*I and *Hind*III were incorporated into the 5'and 3' ends respectively of the synthetic promoter as shown below:

(**Green** font indicates the *gdh* nucleotide sequences and the underlined sequences represent the *gdh* minimal promoter, **purple** font represents the *Acc65*I restriction site and the **red** font indicates the *Hind*III restriction site).

Forward oligonucleotide strand

5'-
TTGAA**AAGCTT**AAGCT**GACCACAAATAACGCCTTTAATTACAGGCGCCCCAGATTTTAAAATG**CCTG**GGTAC**CCCAG -3'

Reverse oligonucleotide strand

5'-
CTGGG**GGTACC**CAGG**CATTTTAAAATCTGGGGCGCCTGTAATTAAAGGCGTTATTTGTGGTC**AGCTT**AAGCTT**TTCAA-3'

Complementary oligonucleotide strands as describe above were annealed by mixing equal concentrations of each oligonucleotide and incubating at 95˚C for 5 minutes, then cooled gradually to room temperature. The annealing efficiency was determined by agarose gel electrophoresis (section 2.1.2) of a single oligonucleotide strand and annealed double oligonucleotide strands using 2.5 % agarose gel at 80 V for 80 minutes.

2.2.2 Digestion of Synthetic Glutamate Dehydrogenase Promoter and Plasmid pTurboRFP

The optimal DNA concentration for visualisation was determined by restriction digestion. Different ranges of DNA concentration were digested with 10 units of *Acc65*I (Promega) and *Hind*III (Promega) together with 1 X Buffer E (Promega) in a total volume of 20 µL. The reactions were incubated at 37 ˚C for 3 hours. Agarose gel electrophoresis was performed as per section 2.1.2 to identify the digested product. The composition of restriction digestion reactions with different concentration of plasmid vector and promoter DNA were set up according to Table 2.2 a & b.

Table 2.2a: Plasmid TurboRFP DNA optimisation

Reaction	1	2	3	4	5	6	7
Plasmid TurboRFP DNA concentration(µg)	0.2	0.5	0.7	1.0	1.2	0.5 (-)	1.2 (-)

(-): negative control

Table 2.2b: Promoter DNA optimisation

Reaction	1	2	3	4	5	6
Promoter DNA concentration(µg)	0.2	0.5	0.8	1.0	0.5 (-)	1.2 (-)

(-): negative control

The optimal DNA concentration of the promoter and the promoterless plasmid TurboRFP were digested with *Acc65*I and *Hind*III with the same conditions as described above, respectively. The desired digested products were then excised from the gel and the DNA extracted using the gel extraction kits as described in section 2.1.3.

2.2.3 Ligation of Synthetic Glutamate Dehydrogenase Promoter into pTurboRFP

The digested synthetic promoter was ligated into the digested promoterless TurboRFP as described in section 2.1.4. Two different insert to plasmid vector molar ratios (3:1 & 8:1) containing 4.8 ng (3:1) or 12.95 ng (8:1) of digested promoter were used, respectively.

2.2.4 Transformation, Selection and Growth of Selected White Colonies, Plasmid Preparation

The ligated product was transformed as per section 2.1.5. White colonies were picked from the LB plate (containing 50 mg/mL kanamycin) and inoculated into individual McCartney bottles, each containing 5 mL LB broth and 50 mg/mL of kanamycin. The cultures were then incubated overnight at 37 ˚C on a gyratory-shaker at 225 rpm. The pRFPneo plasmids from overnight *E. coli* cultures were purified according to section 2.1.7.

2.2.5 Modified Cloning Methods

If no colony was observed, the cloning process was repeated with this modified version. The digestion reaction was performed as described in section 2.2.2 , but the concentration of plasmid vector and promoter were doubled and monitored according to the required concentration of vector and insert ration (3:1 and 8:1). The desired digested plasmid vector and promoter fragments were purified together in the same column as described in section 2.1.3. Out of 20 µL of eluted digested plasmid vector and promoter, 8 µL of eluted DNA was then transferred into new 1.5 mL microcentrifuge tube. The ligation reaction was performed by adding three units of T4 DNA Ligase (Promega) and 1 x ligation buffer into the microcentrifuge that contains 8 µL of eluted DNA. The reaction tube was incubated overnight at 4 ˚C.

2.3 Stable Plasmid pRFPneo Vector Construct

2.3.1 Synthetic Glutamate Dehydrogenase Promoter Construct

The pRFP plasmid described in section 2.2 was further modified by the integration of the synthetic glutamate dehydrogenase promoter upstream of the neomycin resistance gene. The coding sequences of this synthetic promoter were same as that described in section 2.2.1, with the exception that a *Cla*I restriction site was incorporated into the 5' and 3' ends of the synthetic oligonucleotide sequences as shown below: (**green** font indicates *gdh* nucleotide sequences with underlined sequences representing the *gdh* minimal promoter, **blue font** represents the *Cla*I restriction site).

Forward Strand

5'-
TTGAA**AACGAT**AAGCT**GACCACAAATAACGCCTTTAATTACAGGCGCCCCAGATTT TAAAAT**GCCTG**AACGAT**CCCAG -3'

Reverse Strand

5'-
CTGGG**TAGCAA**CAGGC**ATTTTAAAATCTGGGGCGCCTGTAATTAAAGGCGTTATTT GTGGTC**AGCTT**TAGCAA**TTCAA-3'

The complementary oligonucleotide strands were annealed as described in section 2.2.1 and the annealing efficiency determined by electrophoresis as described in section 2.1.2.

2.3.2 Digestion of Synthetic Glutamate Dehydrogenase Promoter and Plasmid pRFP

The annealed synthetic promoter and the pRFP plasmid were digested with *Cla*I. The digestion reactions included 1 µg of *gdh* synthetic promoter / pRFP plasmid, 10 units of *Cla* I (Promega) and 1 x buffer C (Promega) in a total reaction volume of 20 µL. The reaction tubes were incubated at 37 ˚C for 3 hours.

2.3.3 Dephosphorylation of Plasmid pRFP

After the incubation of pRFP plasmid with *Cla*I, 10 units of Thermosensitive Shrimp Alkaline Phosphatase (Promega) was added and the mixture was incubated for a further 15 minutes at 37 ˚C. The reaction was then inactivated in a 74 ˚C water bath for 15 minutes. The synthetic promoter was ligated into digested pRFP (section 2.2.3) and transformed as per section 2.1.5.

2.3.4 Modified Cloning Methods

If no colony was observed from the transformation plates, the cloning process was repeated as described in section 2.2.5.

2.3.5 Colony PCR

White colonies containing the insert were picked from the LB plate and restreaked onto another LB plate (containing 50 mg/mL of kanamycin) on the day before performing colony PCR reactions. A pipette tip was used to pick up a bacterial colony, which was then resuspended in 50 µL of sterile Tris-EDTA (TE) buffer. The mixture was incubated in a 100 ˚C water bath for 15 minutes and centrifuged at maximum speed for 2 minutes. The supernatant was then transferred into another new microcentrifuge tube.

The colony PCR was performed as per table 2.3 using the pTurboRFP forward primer and the promoter reverse primer that was designed from Primer 3 (http://workbench.sdsc.edu/) and as shown below:

pTurboRFP forward primer 5'-AGGCGGAAAGAACCAGCTGTG-3'

Promoter Reverser Primer 5'-TTTAAAATCTGGGGCGCCTGTA-3'

The PCR reaction condition was set up as listed in table 2.3.

Table 2.3: Colony PCR conditions

Components	Working Concentration
Forward Primer	10 pmole/µL
Reverse Primer	10 pmole/µL
Taq/Tth polymerase Buffer	1 X
$MgCl_2$	2 mM
dNTP	1 mM
Taq/Tth polymerase	1.2 units
DNA template	5 µL of centrifuged *E.coli* supernatant
Distilled water was added to the reaction to achieve the total volume of 25 µL.	

The thermal cycling program was set up as follows (Figure 2.4):

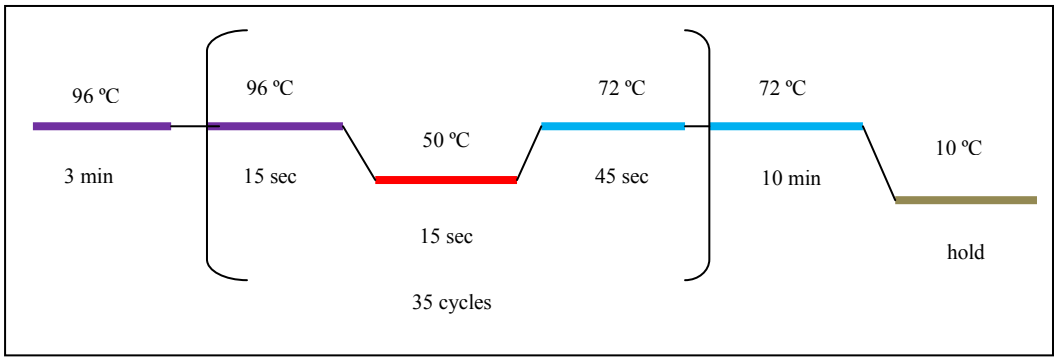


Figure 2.4: Thermal cycling conditions for colony PCR

The amplification products were electrophoresed on a 1.2 % agarose gel (section 2.1.2).

2.3.6 Sequencing

The sequencing of colony PCR product was performed as described in section 2.1.8. The concentration of colony PCR product was determined using NanoDrop and 10 ng of the product was used as template in two separate sequencing reactions, with pTurboRFP forward primer as the forward sequencing primer and reverse primer as reverse sequencing primer. The sequencing results were analysed as per section 2.1.9.

2.3.7 Selection and Growth of White Colonies and Plasmid Preparation

White colonies containing the correctly orientated insert were picked from the LB plate and inoculated into individual McCartney bottles, each containing 5 mL LB broth and 50 mg/mL of kanamycin. The cultures were then incubated overnight at 37 ˚C on a gyratory-shaker at 225 rpm. The pRFPneo plasmids from overnight *E. coli* cultures were purified according to section 2.1.7. This plasmid was named pRFPneo (Figure 2.5)

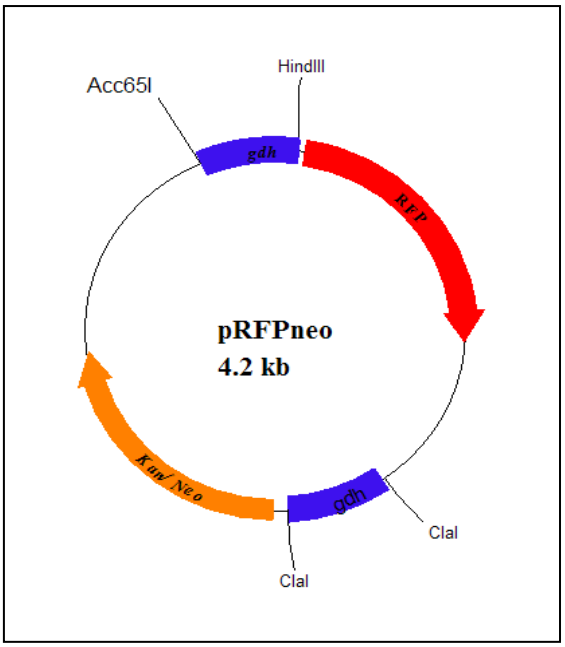


Figure 2.5: Stable plasmid pRFPneo vector

2.4 Stable Plasmid pGFPneo Vector Construct

2.4.1 PCR Amplification

The green fluorescent protein was amplified from plasmid pcDNA6.2/EmGFP-Bsd/V5-Dest (Invitrogen) by PCR using oligonucleotide primers as described below:

eGFP forward primer 5'-ATGGTGAGCAAGGGCGAGGAGC-3'

eGFP reverse primer 5'-CTTGTACAGCTCGTCCATGCCG-3'

The DNA optimisation was performed as described in Table 2.4, the PCR reaction was set up according to Table 2.5 and the thermal cycling conditions were set up as Figure 2.6.

Table 2.4: Plasmid eGFP DNA optimisation

Reaction	1	2	3	4	5	6	7
Plasmid eGFP concentration (ng)	200.0	100.0	50.0	25.0	12.5	6.25	2.0

Table 2.5: eGFP PCR conditions

Components	Working Concentration
Forward Primer	10 pmole/µL
Reverse Primer	10 pmole/ µL
Taq/Tth polymerase Buffer	1X
$MgCl_2$	2 mM
dNTP	1 mM
Taq/Tth polymerase	1.2 units
eGFP DNA template	χ ng
Distilled water was added to achieve the total volume of 25 µL.	

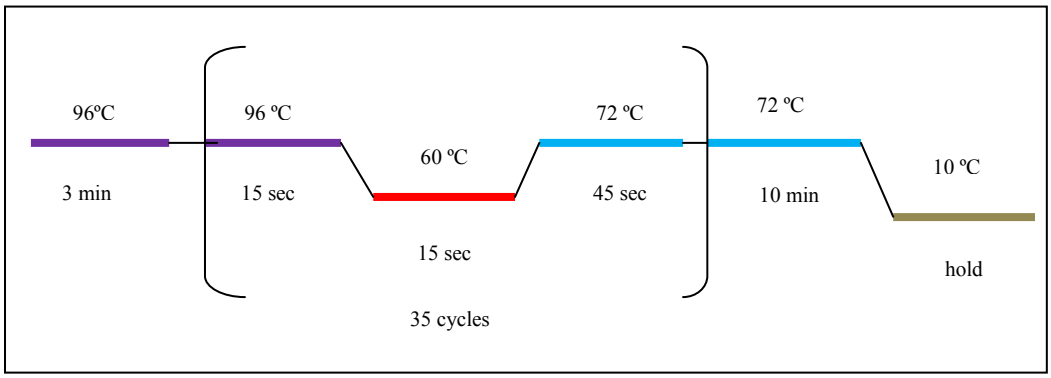


Figure 2.6: Thermal cycling conditions for eGFP PCR reaction

2.4.2 TA cloning of PCR Products and Bacterial Transformation

The ligation reaction was performed as per section 2.1.4. Plasmid pGEM®T- EASY (Promega) plasmid was used as vector in this section.

Two different ratios of insert: plasmid, 3:1 and 8:1, containing 70 ng (3:1) or 186.7 ng (8:1) of eGFP amplicons were used in this ligation reaction. The transformation was then performed as described in section 2.1.5 with the exception that 100 mg/mL of ampicillin (Sigma) was used. The white colonies were grown in LB broth (ampicillin) as described in section 2.1.6. The plasmids were then purified as described in section 2.1.7. The resulting plasmid was named as pGEMT-EASY-eGFP (Figure 2.7).

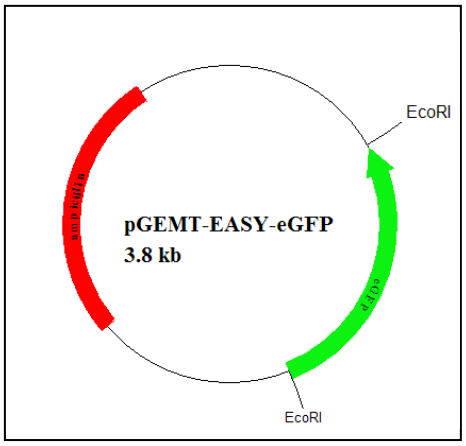


Figure 2.7: Plasmid pGEMT-EASY-eGFP

2.4.3 Plasmid pGEM®-7Zf(+) Subcloning

The pGEMT-EASY-GFP and pGEM®-7Zf(+) (Promega) were digested with *EcoR*I (Promega). The digestion reactions included 1 µg of pGEMT-

EASY-eGFP and pGEM®-7Zf(+), 10 units of *EcoR*I and 1X buffer H (Promega) in a final volume of 20 µL. The reaction tubes were incubated at 37 ˚C for 3 hours. The pGEM7fz (+) was dephosphorylated as per section 2.3.3. The release of eGFP from digested pGEMT-easy-GFP was identified by electrophoresis (section 2.1.2) in a 2.0 % agarose gel and purified as described in section 2.1.3. The insert was subcloned into dephosphorylated pGEM7fz (+) to create pGEM7Zf(+)-eGFP (Figure 2.8) by ligation and transformation as described in sections 2.1.4 and 2.1.5 with the exception that 100 mg/mL of ampicillin was used instead of kanamycin.

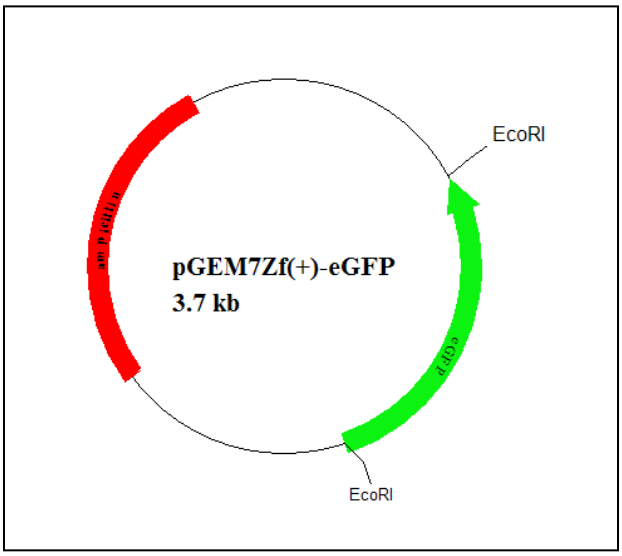


Figure 2.8: Plasmid pGEM7Zf(+)-eGFP

2.4.4 Colony PCR

The white colonies containing the insert were picked from the LB plate and re-streaked onto another LB plate (100 mg/mL of ampicillin). A colony PCR was performed according to section 2.3.4 using the M13 forward primer (5'-GTTTTCCCAGTCACGAC-3') and eGFP reverse primer. The PCR reaction component and thermal cycling were set up as described in Table 2.5 (5 µL of cell suspension) and Figure 2.6.

2.4.5 Sequencing

The sequencing of colony PCR product was performed as described in section 2.1.8. The concentration of colony PCR product was determined with NanoDrop and 20 ng of the product was used as template in two separate sequencing reactions, with M13 forward primer as the forward sequencing primer and eGFP reverse primer as reverse sequencing primer. The sequencing results were analysed as per section 2.1.9.

2.4.6 Selection and Growth of Selected of White Colonies

The pGEM7Zf(+)-eGFP that contained correctly orientated eGFP inserts were picked from the re-streaked plates and grown in LB broth (ampicillin) as described in section 2.1.6. The plasmids were then purified as described in section 2.1.7.

2.4.7 The Digestion of plasmid pRFPneo and pGEM7Zf(+)-eGFP

To assist the replacement of red fluorescent protein of plasmid pRFPneo with eGFP, the eGFP was released from pGEM7Zf(+)-eGFP by digestion with 10 units of *BamH*I (Promega) and 10 units of *Xba*I (Promega) with 1 x buffer C in a final volume of 20 µL, and incubated at 37˚C for 3 hours. Plasmid pRFPneo was also digested with *BamH*I and *Xba*I to remove the red fluorescent protein from base 136 to base 829 of the plasmid. The digestion reaction conditions of plasmid pRFPneo were set up as described for pGEM7Zf(+)-eGFP. The desired digested fragments, 700 bp eGFP and 3580 bp pRFPneo were identified by electrophoresis on a 1.2 % agarose gel at 80 V (section 2.1.2) and purified as described in section 2.1.3.

2.4.8 Ligation, Transformation, Selection White Colonies and Plasmid Purification

The eGFP insert was ligated with digested plasmid pRFPneo as described in section 2.1.4. The ligation products were transformed (Section 2.1.5) and the white colonies were selected and grown as described in section 2.1.6. Plasmid purification was carried out as described in section 2.1.7. The resulting plasmids were named pGFPneo (Figure 2.9).

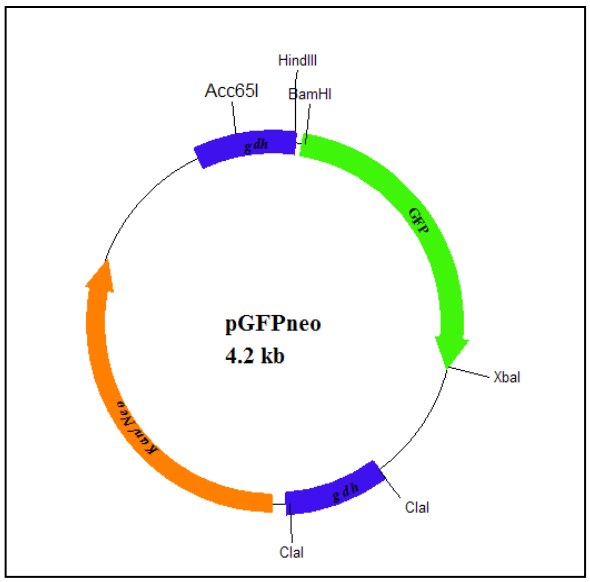


Figure 2.9: Stable plasmid pGFPneo vector

2.5 *Giardia duodenalis* Transfection

2.5.1 Ethanol Precipitation

To perform *G. duodenalis* transfection, 25 µg of pRFP, 100 µg of pRFPneo and pGFPneo were precipitated and resuspended in a total volume of 20 µL to concentrate the plasmid, respectively. The plasmid DNA was precipitated by adding one 0.1 volume of chilled 3 M sodium acetate (pH 5.2) and 2.5 times volume of ice-cold 100 % ethanol, following which the samples were placed at -20˚C for at least one hour. The samples were then centrifuged for 15 minutes at maximum speed in a 4˚C microcentrifuge, and the supernatant decanted. Cold 70% ethanol was added to the sample and centrifuged for 5 minutes at maximum speed in a 4˚C centrifuge. The supernatant was decanted and the residual ethanol was removed by pipetting. The samples were air-dried at room temperature and re-dissolved in 20 µL of distilled water.

2.5.2 Electroporation

2.5.2.1 Transient transfection

Circular plasmid pRFP was electroporated into *Giardia* trophozoites (BAH 3c3 and BAH 34c8) as described previously (Singer, Yee, and Nash 1998; Yee and Nash 1995). The cells were harvested at mid-to-late logarithmic phase by chilling the tube in ice for 30 minutes, a cell count was performed using a haemocytometer under light microscope (model BX 41, Olympus) and the trophozoites resuspended to a final concentration of 3.33×10^7 cells/mL in culture medium. An aliquot of cell suspension and 25 μg of plasmid DNA were transferred into a 0.2 cm electroporation cuvette, and incubated on ice for 5 minutes. The cells were immediately electroporated at 350 V, 1000 μF, and 700 Ω (BTX, San Diego, CA). The cells were then placed on ice for 15 minutes and transferred into 10 mL culture tubes with fresh media (BS-1-33) containing broad spectrum antibiotics (penicillin and streptomycin). Each electroporation was performed in triplicate and a mock transfectant was performed. The growth of trophozoites was monitored microscopically.

After 48 hours recovery period, 100 µL aliquots of the culture were plated onto LB agar plates to monitor for potential bacterial contamination.

2.5.2.2 Stable transfection

Both 100 µg of circular pRFPneo and pGFPneo were independently electroporated into *Giardia* trophozoites (BAH 2c1) as described above (section 2.5.2.1).

2.5.3 G418 Drug Selection

After 24 hours incubation without any drug at 37˚C, 100 µL aliquots of the culture that containing stable transfected trophozoites were plated onto LB agar plates to monitor for potential bacterial contamination. The non-adherent cells were decanted and the culture replenished daily with fresh medium containing 150 µg/ mL of G418 (Sigma) for 4 days.

2.5.4 Drug Sensitivity

The cells were harvested at mid-to-late logarithmic phase by chilling the tube in ice for 30 minutes, and cell count was performed using a haemocytometer and the trophozoites resuspended to a final concentration

of 3.33 x 10^7 cells/mL in culture medium. An aliquot of cell suspension was added into each borosilicate tube containing 10 mL of culture medium and the concentration of drug was set up as described in table 2.6. The culture was inbucated at 37 ºC for 12 hours and then the non-adherent dead cells were decanted. Ten millilitre of 1 x PBS was added into each tube and incubated on ice for 15-30 minutes to detach the alive cells from the flask surface. The tube was mixed by inversion to disperse the trophozoites and a cell count performed using a haemocytometer under a light microscope.

Table 2.6: Drug concentration

Reaction	1	2	3	4	5
Drug concentration (µg/mL)	0	50	100	150	200

2.5.5 Microscopic Analysis

The activity of transfected trophozoites were identified under a fluorescent photomicroscope (model BX 51, Olympus). For each observation, 1µL of cell culture was transferred on to the slide. If the cell density was too high

in each microlitre, a series of dilutions 1:20, 1:50 and 1:100 was performed using distilled water.

Chapter 3

Results

3 Results

3.1 Transient Transfection System

3.1.1 Synthetic Promoter Annealing Efficiency

As the mammalian promoter, SV 40 (simian virus 40), is not functional in *Giardia*, a synthetic 44 bp glutamate dehydrogenase minimal promoter was designed to drive the expression of a fluorescent protein in the trophozoites. The annealing efficiency of complementary oligonucleotide strands was determined by comparing the migration rate between annealed strands (double stranded) promoter and single stranded oligonucleotide using 2.5 % agarose gel electrophoresis.

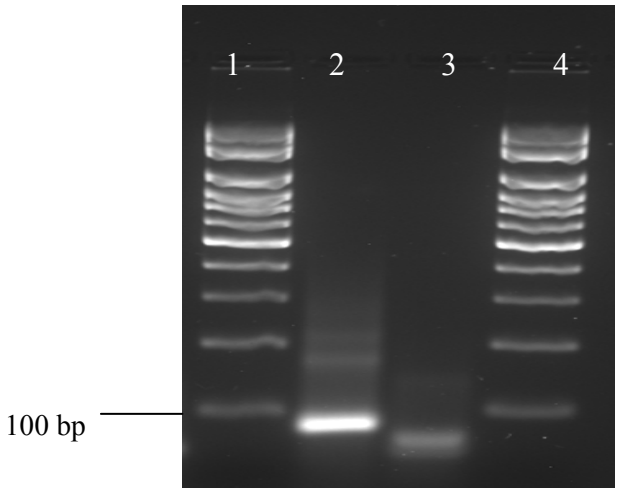


Figure 3.1: Comparing migration rate of annealed double stranded oligonucleotide synthetic promoter and single stranded oligonucleotide
Lane 1: 100 bp ladder DNA marker, Lane 2: annealed double stranded synthetic promoter, Lane 3: single stranded oligonucleotide strand, Lane 4: 100 bp ladder DNA marker

The migration rate of annealed synthetic promoter (Figure 3.1, lane 2) was slower than the single stranded oligonucleotide strand (Figure 3.1, lane 3), suggesting that the complementary oligonucleotide strands had annealed properly and therefore had a higher frictional drag and inefficient movement through the pores of the gel as compared to the movement of single stranded oligonucleotides. In addition, although the gel was loaded with 1 µg of annealed synthetic promoter and single stranded oligonucleotide, the former had a brighter band as compared to the latter, showing that the intercalation of Sybr Safe with single stranded DNA was reduced and therefore not as bright as annealed double stranded DNA.

3.1.2 Transient Plasmid pRFP Vector Construct

Prior to the cloning process, an optimisation of plasmid pTurboRFP DNA and synthetic promoter DNA were performed to determine the optimal DNA concentration required for visualisation.

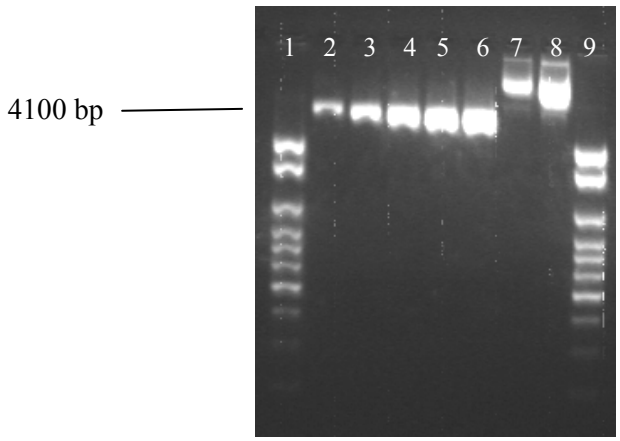


Figure 3.2: Plasmid pTurboRFP DNA optimisation
Lane 1: 100 bp ladder DNA marker, Lane 2: 0.2 µg of plasmid, Lane 3: 0.5 µg of plasmid, Lane 4: 0.7 µg of plasmid, Lane 5: 1.0 µg of plasmid, Lane 6: 1.2 µg of plasmid, Lane 7: negative control of 0.5 µg plasmid, Lane 8: negative control of 1.2 µg of plasmid. Lane 9: 100bp ladder DNA marker

Two fragments, 23 bp and 4.1 kbp would be produced after double digestion. However, the 23 bp fragment was too small to be visualised on 2.5 % gel, only a single distinct bright band was observed in each of the digested plasmid vectors (Figure 3.2, lanes 2 to 6). The brightness of the band increased with respect to their DNA concentrations. The appearance of a faint band below the wells in lanes 7 and 8, indicated the uncut

plasmid structures still maintained in supercoiled form. Once the plasmid was digested with *Acc65*I and *Hind*III, the supercoiled structure was converted to linear form, therefore only one distinct and smaller sized band was present in each lane containing digested plasmid.

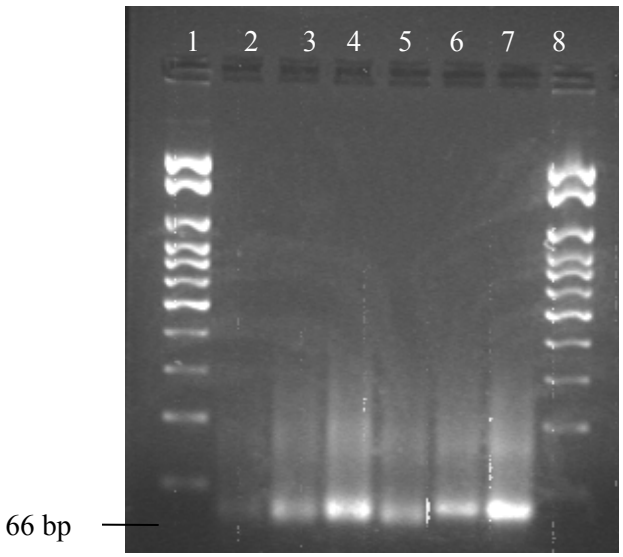


Figure 3.3: Synthetic promoter DNA optimisation
Lane 1: 100 bp ladder DNA marker, Lane 2: 0.2 µg of promoter, Lane 3: 0.5 µg of promoter, Lane 4: 0.8 µg of promoter, Lane 5: 1.0 µg of promoter, Lane 6: negative control of 0.5 µg of promoter, Lane 7: negative control of 1.0 µg of promoter, Lane 8:100 bp ladder DNA marker

The synthetic promoter has a total size of 78 bp and digestion with *Acc65*I and *Hind*III released a 66 bp and two 7 bp fragments. However, the 7 bp fragment was too small to be identified in 2.5 % agarose. Therefore, the digested promoter was compared to the migration of undigested promoter to determine if the digestion had taken place. The result (Figure 3.3)

showed that digested synthetic promoter of 0.5 µg and 1.0 µg produced bands (lanes 3 and 5), which were slightly smaller than their negative controls respectively (lanes 6 and 7).

Based on the results of the DNA optimisation, 1µg of plasmid vector and synthetic promoter were selected to perform the cloning process as described in section 2.2. However, transformation plates showed no colony in both 3:1 and 8:1 ratios. The cloning process was repeated several times yet no colonies were observed. To overcome the problem, the process was repeated as described in section 2.2.5. The transformation plates showed approximately 50 colonies at both the 3:1 and 8:1 ratio. Due to directional cloning being used, colony PCR was not deemed necessary.

3.1.3 Transient Fluorescence Expression

To determine whether the insertion of the glutamate dehydrogenase minimal promoter upstream of the red fluorescent protein is able to drive the expression of red fluorescent protein in trophozoites, a transient transfection was performed as described in section 2.5.2.1. The activity of transfected trophozoites were examined 48 hours after electroporation using a fluorescence photomicroscope. Approximately 5 % of assemblage B trophozoites displayed a red fluorescence, indicating a low transfection

efficiency and no red fluorescence was seen in assemblage A. No cell density difference was found between the transfected trophozoites and mock transfectant. However, the time required to reach confluency in transfectant and mock transfectant was increased as compared to untransfected cells, indicating a recovery process from electroporation. Figure 3.4 shows one of the red fluorescent trophozoites with prominent nuclei and median bodies.

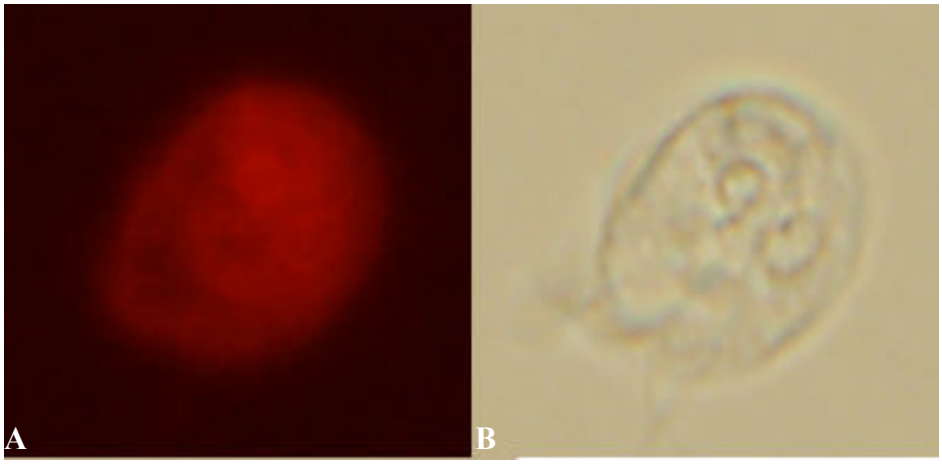


Figure 3.4: The red fluorescent trophozoites (A) and its brightfield observation (B) of assemblage B

Localised fluorescence was not found in transfected trophozoites indicating that the glutamate dehydrogenase minimal promoter was sufficient to drive a diffusible expression of red fluorescent protein in trophozoites. Most of the red fluorescent trophozoites' nuclei and median bodies could be seen vividly. No bacteria or fungus contamination was

observed on the LB plates suggesting that the fluorescent expression was not an artifact.

Due to the low transfection efficiency, the transfected trophozoites were further subcultured (section 2.1.1). However, the identification of red fluorescence was greatly hindered by the massive population of untransfected *G. duodenalis*. Therefore, instead of observing the trophozoites activity from 1 µL of undiluted culture medium, different dilutions (1:10, 1:20, 1:50, and 1:100) were performed to assist in the identification of red fluorescent trophozoites yet no additional red fluorescent trophozoites could be identified.

3.2 Stable Transfection System

3.2.1 Stable Plasmid pRFPneo Vector Construct

The glutamate dehydrogenase minimal promoter was shown to be able to drive high expression of red fluorescent protein in transient transfection. To select the transfected cells, the glutamate dehydrogenase minimal promoter was cloned upstream of neomycin as described in section 2.3.

Due to the use of same plasmid (pRFP) and identical promoter size as described in section 2.2, no DNA optimisation was performed in either glutamate dehydrogenase minimal promoter and pRFP plasmid. The initial cloning process yielded no colonies. Therefore, the methods described in section 2.2.5 were applied. Colony PCR was performed to screen for the correctly orientated promoter insertion.

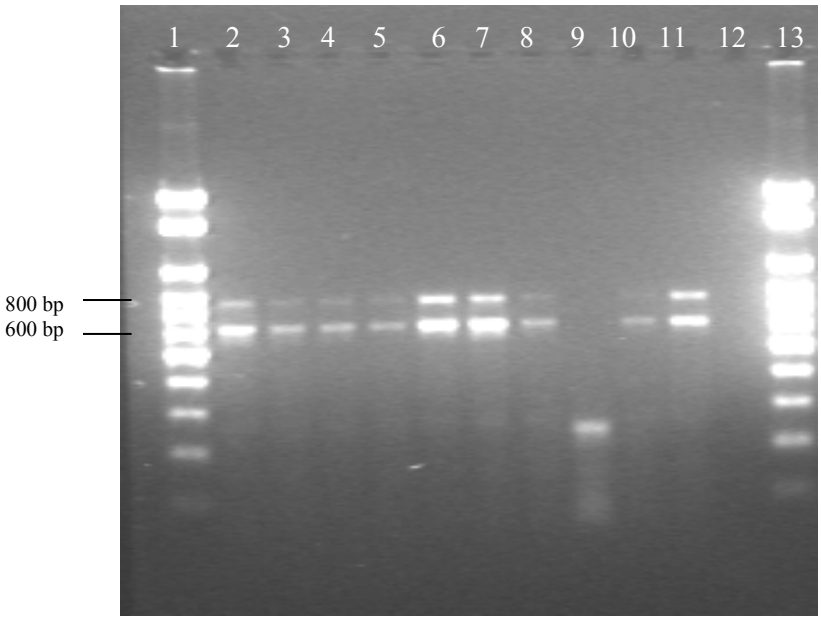
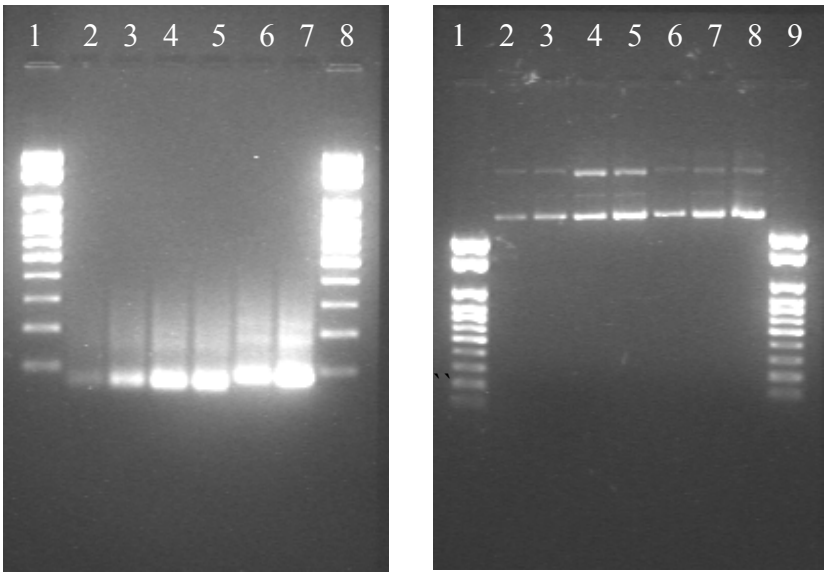


Figure 3.5: Colony PCR screening for correct orientation inserted synthetic promoter
Lane 1: 100 bp ladder DNA marker, Lane 2-11: recombinant cells containing insert, Lane 12: PCR negative control, Lane 13: 100 bp ladder DNA marker

However, no expected PCR product was observed (400 bp) from Figure 3.5. As a further complication, multiple bands (800 bp and 600 bp) were produced in most colonies. This was thought to be due to non-specific binding of the primers since the product was amplified at low annealing temperature. To overcome this problem, the colony PCR reaction was repeated with a higher annealing temperature of 56 °C on the same colonies. However, a 400 bp product was not identified and non specific bands were absent.

DNA optimisation was then performed on both the glutamate dehydrogenase promoter and plasmid pRFP to identify the possible problem as described in section 2.2.2.



(a) (b)

Figure 3.6a & b: DNA optimisation of *Cla* I digestion of synthetic promoter and pRFP plasmid

(a) Lane 1: 100 bp ladder DNA marker, Lane 2: 0.2 µg of promoter, Lane 3: 0.5 µg of promoter, Lane 4: 0.8 µg of promoter, Lane 5: 1.0 µg of promoter, Lane 6: negative control of 0.5 µg of promoter, Lane 7: negative control of 1.0 µg of promoter, Lane 8: 100 bp ladder DNA marker

(b) Lane 1: 100 bp ladder DNA marker, Lane 2: 0.2 µg of plasmid pRFP, Lane 3: 0.5 µg of plasmid pRFP, Lane 4: 0.7 µg of plasmid pRFP, Lane 5: 1.0 µg of plasmid pRFP, Lane 6: 1.2 µg of plasmid pRFP, Lane 7: negative control of 0.5 µg of plasmid pRFP, Lane 8: negative control of 1.2 µg of plasmid p RFP, Lane 9: 100 bp ladder DNA marker

Promoter DNA optimisation (Figure 3.6a), showed that the digested promoter (lanes 4 and 5) had a smaller band then the undigested plasmid (lanes 6 and 7) indicating the efficiency of *Cla*I digestion. On the contrary, no size difference was found between the digested (Figure 3.6b, lanes 2-6) and undigested pRFP (Figure 3.6b, lanes 7 and 8). The cloning process was repeated several times yet no correct digestion fragment was identified. It was suspected that methylation was blocking the *Cla*I site, hence the plasmid could not be digested. Therefore, the ligation and subsequent procedures were not be possible.

To remove the methylation-blocking site, the pRFP was transformed into dam$^-$/dcm$^-$ *Escherichia coli* cells (*New England* Bioloab, US) instead of JM109 cells, before proceeding to the cloning process. Following cloning into the new cell line, a plasmid preparation was performed and the plasmid (1 µg) re-tested for *Cla*I digestion (Figure 3.7).

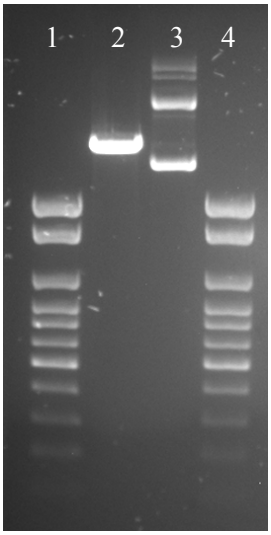


Figure 3.7: Digestion of *Cla*I
Lane 1: 100 bp ladder DNA marker, Lane 2: 1µg of digested plasmid, Lane 3: negative control of undigested plasmid, Lane 4: 100 bp ladder DNA marker

Figure 3.7 shows that only one distinct fragment was produced following *Cla*I digestion (lane 2) as the undigested plasmid (lane 3) still maintained its supercoiled structure which was indicated by the presence of multiple banding. Due to the repeating failure of *Cla*I digestion process, cloning was repeated several times. Finally, transformation plates showed approximately 120 white colonies which were screened by colony PCR as per section 2.3.5 for the presence of correct orientation of inserts, using the pTurboRFP forward primer and promoter reverse primer.

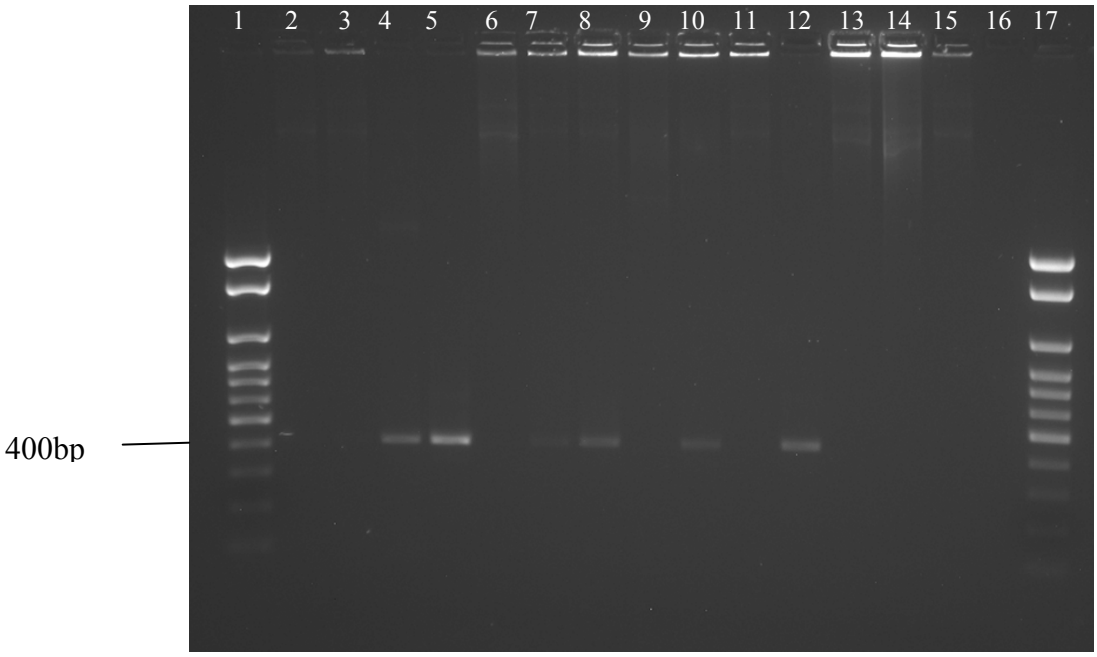


Figure 3.8: Colony PCR screening for correct orientation inserted synthetic promoter

Lane 1: 100 bp ladder DNA marker, Lane 2-15: recombinant cells containing insert, Lane 16: PCR negative control, Lane 17: 100 bp ladder DNA marker

Several colonies possessed inserts of approximately 400 bp (Figure 3.8). The colonies that contained correctly orientated inserts were sequenced and aligned with the original expected product sequence. The alignment showed pair-wise identity matches of 99%. This demonstrated that the glutamate dehydrogenase minimal promoter had been inserted upstream of the neomycin resistance gene. The whole plasmid sequences of pRFPneo are shown in appendix I.

3.2.2 Stable Plasmid pGFPneo Construct

TA cloning was performed to amplify the eGFP fragment from a commercial green fluorescent protein plasmid. PCR DNA optimisation for the eGFP primer pair was performed.

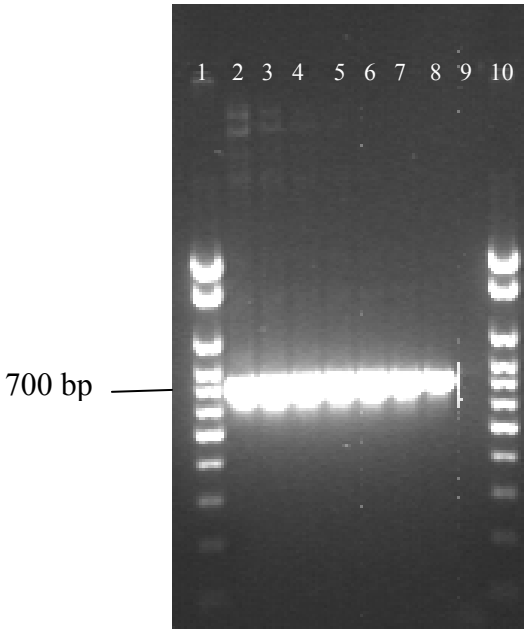


Figure 3.9: PCR DNA optimisation
Lane 1: 100 bp ladder DNA marker, Lane 2: 200ng of plasmid, Lane 3: 100 ng of plasmid, Lane 4: 50 ng of plasmid, Lane 5: 25 ng of plasmid, Lane 6: 12.5 ng of plasmid, Lane 7: 6.25 ng of plasmid, Lane 8: 2 ng of plasmid, Lane 9: PCR negative control, Lane 10: 100 bp ladder DNA marker

A distinct bright band at approximately 700 bp was visible in every lane of the gel (Figure 3.9). However, two other faint bands below the well were seen in the reaction containing 200 ng (lane 2) and 100ng (lane 3) of template DNA. This indicates that the DNA concentration was too high

and the supercoiled plasmid structure used as the template was visible during electrophoresis. To avoid complications, 2 ng of DNA was selected as the optimal DNA concentration for the amplification of the 700 bp eGFP. The amplicon was purified from the gel and cloned into pGEM®-T EASY. Transformation yielded several white colonies which were further screened by PCR for the presence of inserts, using eGFP forward primer and reverse primers. This plasmid was referred to as pGEMT-EASY-eGFP.

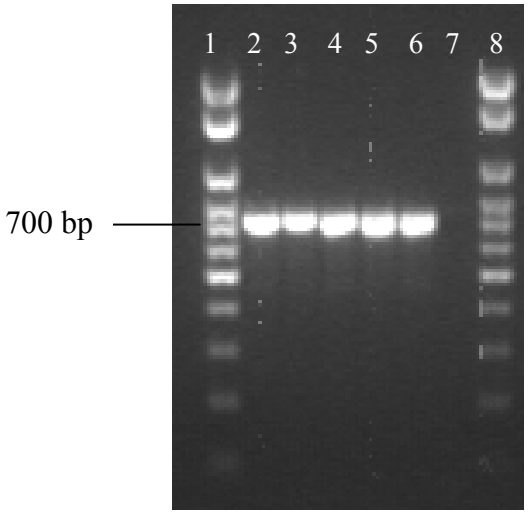


Figure 3.10: White colony screening for correct inserts
Lane 1: 100 bp ladder DNA marker, Lane 2-6: recombinant cells containing insert, Lane 7: PCR negative control, Lane 8: 100 bp ladder DNA marker

A bright distinct 700 bp amplicon of eGFP in each lane was observed (Figure 3.10). A sequencing reaction was performed on the positive

samples, with the alignment between the original sequence and pGEMT-EASY-eGFP showing a 100 % pairwise identity.

The plasmid pGEMT-EASY-eGFP was then subcloned into pGEM®-7Zf(+) to provide a convenient digestion site for cloning into pRFPneo. There were approximately 100 white colonies, which were screened for the presence of correctly orientated inserts using colony PCR reaction as shown in figure 3.11.

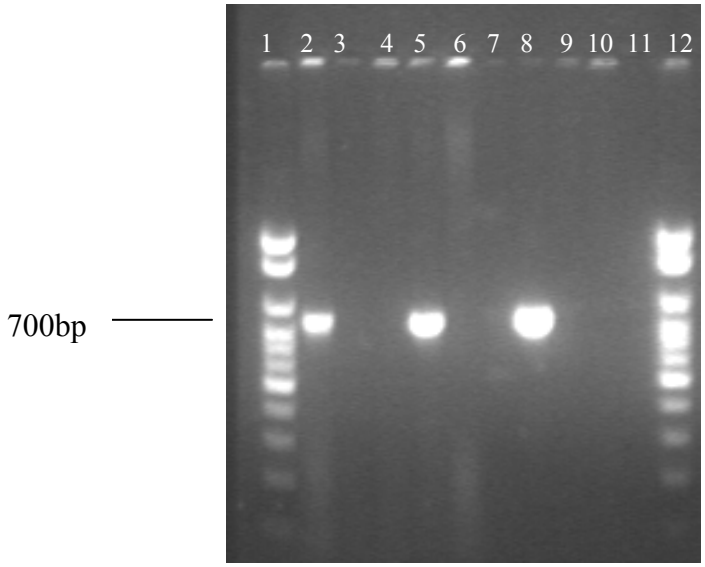


Figure 3.11: Colony PCR screening for correct orientation inserted eGFP
Lane 1: 100 bp ladder DNA marker, Lane 2-11: recombinant cells containing insert, Lane 12: PCR negative control, Lane 12: 100 bp ladder DNA marker

Out of 10 randomly selected colonies, only three colonies showed a distinct bright band at 700 bp, indicating an insert of correct size and orientation (Figure 3.11, lanes 2, 5, and 8). Alignment was also performed

between the colonies and the expected sequence, a 100 % pairwise identity being obtained. This new plasmid was termed pGEM-7Fz(+)-eGFP.

In order to replace the red fluorescent protein of pRFPneo plasmid with green fluorescent protein of pGEM7Zf(+)-eGFP, the 700 bp eGFP fragment was cloned into the 3kbp pRFPneo large fragment as described in section 2.4.7 and section 2.4.8. The presence of the eGFP fragment in the pRFP plasmid was determined by colony PCR reaction (Figure 3.12).

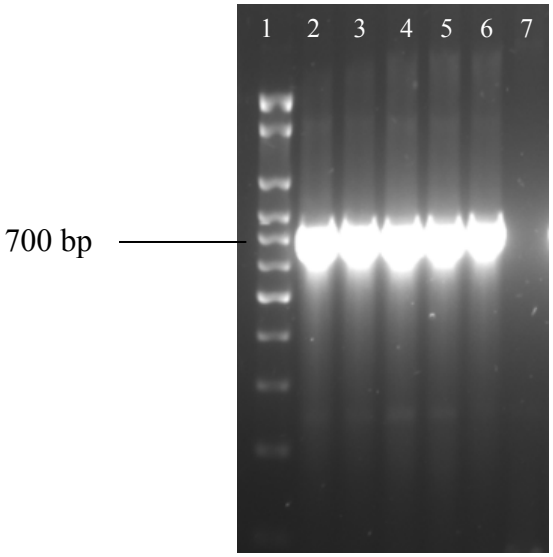


Figure 3.12: White colony PCR screening for inserts eGFP
Lane 1: 100 bp ladder DNA marker, Lane 2-6: recombinant cells containing insert, Lane 7: PCR negative control

All the randomly selected white colonies yielded PCR products of approximately 700 bp and were sequenced. The alignment showed that the amplicon was a 99% match to the expected sequence. This plasmid is

referred to as pGFPneo. The whole plasmid pGFPneo sequences are shown in appendix II.

3.2.3 Stable Fluorescence Expression

Stable transfection was performed not only to select for transfected cells but also to maintain the plasmid episomally under G418 drug selection for a long period. In order to obtain a higher percentage of fluorescent trophozoites, 100 μg of pRFPneo and pGFPneo were transfected into the trophozoites in assemblage A but not assemblage B. This is because due to time constraints and the fact that previous studies have shown that stable cell line could be developed in assemblage A (Su et al. 2007; Sun, Chou, and Tai 1998; Yu, Wang, and Wang 1996), therefore only assemblage A was applied in this section. Due to previous failure of transient fluorescent expression, a different clone of assemblage A (BAH 2c1) was used.

Both pRFPneo and pGFPneo were transfected as described in section 2.5.2.2 and were selected using G418 as described in section 2.5.3. However, despite no cell count being performed, it was identified that the number of trophozoites decreased daily with some dead trophozoites being observed. At day 4, no trophozoites were found to have survived. Contrary to this, the cell density of mock transfectant was increasing as compared to

the transfected cells. The transfection was repeated several times, yet all the trophozoites were dead by day 4.

Initially, it was suspected that the G418 concentration of 150 mg/mL drug was too high and transfected trophozoites were too sensitive to this concentration. To identify the LD_{50} (lethal dosage that killed 50 % of population) of *Giardia* to G418, a series of different drug concentrations were added to untransfected cells. A control into which no drug was added was used to determine the *Giardia* survival rate after 12 hours following drug inoculation. The survival rate of the control was assumed to be 100%.

Table 3.1: The survival rate of untransfected trophozoites after exposure to different drug concentrations

Drug concentration	Survival Rate %
0 µg/mL	100 %
50 µg/mL	44 %
100 µg/mL	34 %
150 µg/mL	21 %
200 µg/mL	16 %

As shown in table 3.1, the survival rate of trophozoites was 44 % when 50 µg/mL of G418 was added, but when the concentration was increased up to 200 µg/mL, the survival rate was only 16 %. The transfection was repeated and the transfected trophozoites were selected using a lower concentration of 50 mg/mL of G418. However, the trophozoites were still unable to survive up to day 4.

Chapter 4

Discussion

4 Discussion

4.1 General Overview

The modified mammalian fluorescent expression system has been previously shown to be successful in detecting sexual reproduction in trypanosomes *in vivo* (Gibson et al. 2006; Gibson and Whittington 1993). Consequently, the same approach was used to observe potential sexual exchange in *G. duodenalis*. The advances in transfection and expression of exogenous genes in *G. duodenalis* have opened up the possibility of probing the existence of sexual reproduction in the life cycle. Before looking into the possibility of sexual exchange in *G. duodenalis*, both transient and stable transfection of mammalian fluorescent expression in trophozoites have to be established.

4.2 Transient Transfection

4.2.1 Cloning Methods

In our system, the 44 bp glutamate dehydrogenase minimal promoter was used to drive the expression of a promoterless commercial RFP. During the cloning process, it was found that using a conventional cloning technique (section 2.1) was not successful in this study. This might have been due to the small size of the promoter and therefore 1: 3 and 1:8 ligation ratios were insufficient to ligate to 100 ng of plasmid. Furthermore, using high concentrations of promoter DNA will enhance the occurrence of self ligation between promoters. Thus, a modified cloning technique as described in section 2.2.5 was applied to overcome this problem. More than 30 colonies were obtained which indicated the modified cloning was suitable to clone a small fragment. This may be due to the co-purification of the digested synthetic promoter and plasmid vector leading to an increased interaction between the digested promoter and plasmid, therefore improving the ligation reaction.

4.2.2 Fluorescent Trophozoites

The presence of red fluorescent trophozoites in assemblage B (BAH 34c8) indicates that DNA-based transient transfection was possible in *Giardia* and the possibility of artifactual transfection of contaminating bacteria or fungi was very unlikely. In the case of the latter, stippled or localised fluorescence would have been expected due to the abundance of the smaller size of bacteria. In addition, two broad-spectrum antibiotics (penicillin and streptomycin), were used in the culture medium after and before electroporation to prevent bacterial contamination. The potential of fungal contamination was also monitored microscopically. Furthermore, the electroporation conditions of *Giardia* were not suitable for transforming bacteria since a smaller capacitance and a higher voltage would be required (Yee and Nash 1995). Similarly with fungus, which is more difficult to transform, not only because of the electroporation conditions, but also because their cell wall structure contains high amounts of mannoproteins and glucans that need to be enzymatically removed before electroporation (Thompson et al. 1998; Zlotnik et al. 1984).

4.2.3 Fluorescence Expression Pattern

The fluorescence within the trophozoite not only shows a diffusible fluorescence expression pattern but both nuclei and median body were fluorescing. This was because in our construct, a signal sequence such as a short N and C terminal amino acid was not included in our vector construct to direct the protein to undergo a further post-translational translocation after protein synthesis (Lewin 2006). Post-translational translocation is the movement of a protein across a membrane after it has been released into the cytosol (Lewin 2006). To direct protein into the nucleus, the nuclear protein must have a nuclear localization signal that enables them to pass though the nuclear pores (Becker, Kleinsmith, and Hardin 2003). Nuclear localisation signals usually contains 8-30 amino acids, which include proline, lysine and arginine amino acid sequences on the vector (Becker, Kleinsmith, and Hardin 2003). A study has suggested that a nuclear localization signal with additional Pro-Lys-Lys-Lys-Arg-Lys-Val amino sequences in the plasmid was sufficient to drive a nuclear location signal in the cell (Kalderon et al. 1984). Since no signal sequences were included in our construct, after the synthesis of red fluorescent protein from the ribosome, it was released into the cytosol.

Therefore, a diffusible fluorescent expression was observed in the trophozoites.

4.2.4 Glutamate Dehydrogenase Minimal Promoter

In our study, the fluorescent trophozoites also showed that the glutamate dehydrogenase minimal promoter was able to drive the expression of red fluorescent protein in a mammalian plasmid. The minimal promoter consisted of an A/T rich region and CAAT box (g-CAB element). The A/T rich region was used to position RNA polymerase II for the correct initiation of transcription while the g-CAB element was bound by a family of transcription factors which up-regulate transcription of the gene (Yee et al. 2000). Our studies agree with the study by Yee et.al (1995) where the reporter gene activity was inversely proportional to the amount of glutamate dehydrogenase coding region remaining in the transfected plasmid (Yee and Nash 1995). Since no glutamate dehydrogenase coding gene was included in this construct, it may have further ensured the correct folding of red fluorescent protein without any other coding sequence interference (Yee and Nash 1995). Therefore, this study supports the fact that the 44 bp promoter was all that was required to maintain a fully functional reporter gene.

4.2.5 Absence of Fluorescence Expression in Assemblage A

The absence of fluorescence in assemblage A contradicted the study by Singer et al. (1998) that showed assemblage A isolates were able to maintain the circular transfected DNA episomally. In contrast, assemblage B did not replicate episomally. This is because assemblage B was more restricted to which sequences function as origins of DNA replication while assemblage A can utilise any cryptic origin to replicate episomally (Singer, Yee, and Nash 1998).

To our knowledge, this is the first transient expression in assemblage B isolates of *G. duodenalis*. All published data transfected the plasmid exclusively into WB reference isolates (assemblage A) to facilitate gene analysis studies. However, no transfection study has been performed on assemblage B isolates due to their inability to replicate episomally (Sun, Chou, and Tai 1998; Sun, Su, and Gillin 2005; Sun and Tai 2000; Yee and Nash 1995; Yu, Wang, and Wang 1996). Therefore, our study has demonstrated that a transient expression is possible in assemblage B isolates.

The expression of red fluorescent protein in assemblage B may also be due to the integration of plasmid into their *Giardia* genome via non-homologous recombination between the plasmid and genomic DNA. Homologous recombination in this study was unlikely to have occurred as the plasmid is required to be flanked by a high number of coding sequences (Wu, Kirkman, and Wellems 1996) and our construct only involved a short minimal promoter sequence. This may indicate that the use of a minimal promoter or short *Giardia* coding sequences were sufficient to direct non-homologous recombination in trophozoites. However, pulse field gel electrophoresis and Southern blotting should be performed to determine if the plasmid was maintained episomally or integrated.

Since the assemblage A isolate is able to maintain the plasmid episomally, the reason that no fluorescent trophozoites were observed in our study is not known but it is highly suspected that this was due to the failure of transfection.

4.2.6 Electroporation Conditions

Transient transfection was performed as described by Yee el al. (2000) with the exception that only 700 Ω resistance was used instead of 720 Ω as the device could not be set to 720 Ω. It is not known whether the minor change in resistance had reduced the transfection efficiency as only 5 % of red fluorescent trophozoites were identified in the assemblage B isolate and none in the assemblage A isolate 48 hours after electroporation. However, as no comparison of transfection efficiencies could be made between our study and others, it is not known whether such low transfection efficiency was a common scenario in transfected cells.

Molecular uptake and cell viability after electroporation is dependent on electrical field strength, energy/voltage, pulse length, number of pulses, cell size and shape plus cell and solute concentration (Canatella et al. 2001). Cell membranes of trophozoites act as electrical capacitors (Dawson et al. 2008) and according to Ohm's Law (Figure 4.1), the voltage increases proportionally to the resistance.

Although it was very unlikely that the minor change of 20 Ω difference would affect the transfection efficiency, a study by Deloerme (1989) showed voltage plays a crucial role during electroporation as the voltage must be high enough to create a pore in the cell membrane, yet low enough to avoid excessive cell death. Therefore, the decrease in resistance may also decrease the voltage required to create a pore in the trophozoites. Hence, out of 10^7 cells, only 5 % of assemblage B trophozoites expressed red fluorescence. No fluorescent trophozoites were detected in assemblage A isolates, which may also suggest that the optimal voltage varies from isolate to isolate.

4.2.7 "Outcompeted Phenomenon"

Transient transfectants are also known as unstable transfectants and it is highly unstable as the transfected DNA is maintained extrachromosomally in the absence of selective pressure (Clark 2005). It is not known whether the absence of red fluorescent trophozoites after 7 days cultivation was due to the instability of the plasmid or the transfected trophozoites were outcompeted by the untransfected trophozoites.

Transient transfection without drug selection results in a heterogeneous population of transfected and untransfected trophozoites. This was of

considerable concern for our study since no red fluorescent trophozoites could be identified after 7 days *in vitro* cultivation. In this study, the untransfected trophozoites may have outcompeted transfected trophozoites, which further hindered the selection process to select the fluorescent trophozoites. The "outcompeted phenomenon" and the display of instability in transient transfection suggested that transiently transfected trophozoites may not be suitable to perform the *Giardia* mixing events unless trophozoites have been transfected with a readily integrated plasmid and could be selected with an appropriate selection method.

4.3 Stable Transfection

4.3.1 Drug Sensitivity

In order to achieve a homogeneous population of transfected cells, stable transfection was performed to maintain the episomal vector plasmids under continuous G418 drug selection. Most stable cell lines are established by initiating the drug selection using 150 mg/mL for the first 4 days post transfection and increasing up to 650 mg/ mL for the rest of the selection period (Su et al. 2007; Sun, Chou, and Tai 1998; Yu, Wang, and Wang 1996). However, in this study, a stably transfected cell line could

not be established under the same conditions. It was suspected that the isolate (BAH 2c1) was too sensitive to this drug concentration (150 mg/mL). As if the drug concentration is too high, the addition of G418 to the culture caused a conformational change in ribosomal RNA and disturbed codon-anticodon recognition at the aminoacycl terminus of aminoacycl- tRNA, hence disrupting the high-fidelity genetic code reading (Eustice and Wilhelm 1984; Su et al. 2007). This may result the death of the trophozoites.

Therefore, drug sensitivity was then performed to identify the LD 50 (lethal dosage) for this isolate. It showed that 56 % of untransfected trophozoites were unable to survive after 50 µg/mL of G418 selection for 12 hours and 80 % growth inhibition after 150 µg/mL and 400 µg/mL drug selections. Hence, after the first attempt using 150 µg/ml of G418, subsequent selection was only performed with 50 µg/mL, yet no cells were able to survive after 4 days of drug selection. This indicated that the failure to develop a stable cell line might be due to the failure of transfection, the plasmid vector construction (Section 4.3.2) or the displayed of "loneliness phenomenon" (Section 4.3.3).

4.3.2 Vector construct

In our study, it was very unlikely that the death of trophozoites was due to the poor expression of the neomycin gene. Our study used the same promoter to regulate the expression of neomycin and fluorescent protein separately, unlike the bicistronic approach where the expression of two proteins are regulated by the same messenger RNA, resulting in poor expression of genes downstream (Yu, Wang, and Wang 1996).

To our knowledge, our study was also the first to construct an expression plasmid whereby the same promoter regulates co-expression of heterologous genes. From the perspective of gene regulation, the function of the promoter should not be affected by the coding sequences in the reporter gene (Lewin 2006). In addition, since the glutamate dehydrogenase promoter was able to drive the highly effective expression of red fluorescence protein, it should also drive a similar level of neomycin resistance expression. Furthermore, as compared to other *Giardia* promoters such as α2-giardin, actin, and ran, glutamate dehydrogenase is a relatively strong promoter while ran promoter is the weakest promoter (Elmendorf et al. 2001). Up until now, the expression of the neomycin gene in a successfully developed stable cell line was

induced by the ran promoter (Liu et al. 2005; Sun, Chou, and Tai 1998). Therefore, the glutamate dehydrogenase promoter should express a better neomycin expression than the ran promoter.

The failure or the weak expression of the neomycin gene in assemblage A isolate transfected with either pGFPneo and pRFPneo may have been due to the fact that for effective neomycin expression, both the 5' and 3' flanking sequence of glutamate dehydrogenase are necessary (Elmendorf et al. 2001; Sun, Chou, and Tai 1998; Yu, Wang, and Wang 1996). However, this does not seem to be the case for the reporter gene where only the 5' flanking promoter is required as shown in our study and others previous studies (Elmendorf et al. 2001; Sun, Chou, and Tai 1998; Yu, Wang, and Wang 1996). The exact reason for this is not known, and further investigation is required.

4.3.3 "Loneliness Phenomenon"

Our study was not in agreement with most studies where no drug resistant cell line was obtained in assemblage A (Su et al. 2007; Sun, Chou, and Tai 1998; Yu, Wang, and Wang 1996). However, the continued observation of some trophozoites at day 2 or 3 of drug selection may suggest that some of trophozoites were able to replicate the plasmid episomally and produce

some neomycin expression. Due to the low transfection efficiency and the effect of the "loneliness phenomenon" where too few trophozoites are present to established a continuous culture (Meloni, Lymbery, and Thompson 1988). Hence, no trophozoites were identified after day 4 of drug selection.

4.4 Limitations and Improvements

Although our study failed to develop a stable drug resistant cell line, based on other successful stable transfection studies, the stable cell line could be maintained episomally under drug selection for more than 6 months, making it a potentially good model for a short-term study (Sun, Chou, and Tai 1998). However, for a long-term study, this model is not feasible as the episomal plasmid may eventually be lost in the population. Therefore, an integrated plasmid for both assemblages A and B isolates is required to facilitate the mixing population study. In addition, it is also necessary to ensure that wild type alleles are not affected by plasmid integration, hence a homologous recombination or site specific recombination is required.

A previous study (Singer, Yee, and Nash 1998) has shown that assemblage A can accept and integrate the linear form of a plasmid into its

genome while assemblage B can accept and integrate either the circular or linear form of a plasmid via homologous recombination. The circular plasmid may be integrated into assemblage B isolates (Singer, Yee, and Nash 1998) by homologous recombination via the production of pseudo-diploids similar to that seen in yeast (Hinnen, Hicks, and Fink 1978), protozoan parasite *Toxoplasma gondii* (Donald and Roos 1994) and *Plasmodium falciparum* (Crabb and Cowman 1996). However, the difference in integrated plasmid mechanisms between assemblage A and assemblage B should be clarified since our study has shown a transient expression in an assemblage B isolate.

With a low transfection efficiency, the effect of the "loneliness" and "outcompeted" phenomena of both transient and stable transfection can be avoided by using an alternate selection method such as microdrop (Binz et al. 1991) or flow cytometry (Hsu et al. 2005). These two techniques are able to sort out the low number of successfully transfected cells from non-transfected cells individually. After sorting the individual fluorescent trophozoites, the small amount of transfected trophozoites can be used to initiate a clonal population under a small volume of culture media.

4.5 Future Aspects

4.5.1 Culture Media Conditions

If a stable fluorescent cell line was successfully developed, the mixing event should be trialled varying different culture conditions. It has been proposed that environmental changes are more likely to impose strong, directional selection on asexually reproducing organisms as the asexual organism may not have enough selectable gene variability to keep up with the changes (Crow 1992). However, through occasional sexual reproduction, it may potentially generate greater variance via recombination and create novel genotypes to keep up with the environmental changes.

Under selection pressure, occasional sexual reproduction also permits the population to explore genotypes that were not represented in the previous generations, thus allowing the population to respond effectively to the selective pressure (Hurst and Peck 1996). It has been demonstrated that occasional sexual reproduction was very effective at increasing the rate of incorporating the beneficial allele and bringing the advantageous alleles at different loci (independent assortment of chromosomes or crossing over in

meiosis) on a single chromosome to counteract the selection pressure (Hurst and Peck 1996). Therefore, it is unlikely to happen in an asexually reproducing organism as it lacks meiosis. Moreover, an extreme change in the environment not only disrupts normal development but may also exert strong phenotypic selection by inducing large phenotypic changes through genetic exchange under selection pressure (Badyaev 2005).

Therefore, the fluorescent *Giardia* should not only be mixed under normal culture conditions, but also under different culture conditions such as changing the bile and cysteine concentrations, adding anti-giardial drugs such as albendazole and chloramphenicol or exposing the culture to different nitrasative agents such as sodium nitroprusside and Roussin's black salt, which possess some antigiardial effects (Lindley, Chakraborty, and Edlind 1988; Lloyd et al. 2003; Farthing, Keusch, and Carey 1985).

In a stable transfection study, Su et al. (2007) also demonstrated that the drug selection (G418 or puromycin) was sufficient to change the metabolism, protein folding, and differentiation status in *Giardia* since cyst formation was identified during the vegetative growth stage. This study had also suggested that *Giardia* cell differentiation could respond quickly to different environments, which may increase the chance of sexual exchange. In addition, the mixing events should also be performed

with genetically distinct organisms as competition between such organisms may also allow within-species variation and the tendency to reproduce sexually (Hurst and Peck 1996).

4.5.2 *In vivo* Study

The presence or the absence of recombination under experimental conditions does not indicate that it will not occur in nature. *In vitro* culture lacks a host immune defense system including antibodies, neutrophils and cytokines, which may impose a selection pressure on *Giardia*. In addition, most *in vitro* culture was performed at 37 ºC. However, a study has shown that during an infection, the host body temperature becomes elevated (Mackowiak, Marling-Cason, and Cohen 1982). Furthermore, different hosts have different parasitic loads and under experimental conditions, inoculum size is customised to a certain concentration to facilitate the study. Moreover, the presence of other parasites and bacteria in the host body may also impose a different selection pressure or "competition" between the species.

There are many other *in vivo* conditions which cannot be mimicked *in vitro* such as the host ionic condition and health conditions which may have contributed to different results of the study. Therefore, *in vivo* studies

should be performed to determine how frequently recombination occurs in nature and whether a host is necessary to allow genetic exchange.

4.6 Conclusion

This study was successful in developing a transient mammalian fluorescent expression system in *Giardia* trophozoites belonging to assemblage B. The successful fluorescent expression in trophozoites indicates that the glutamate dehydrogenase promoter was able to induce the reporter gene in the trophozoites. Although no stable fluorescent system was developed in this study, it has provided some important information on the improvements that can be made to the vector system for future studies. The study also showed that assemblages A and B required different transfection conditions to express the exogenous gene. Furthermore, the study not only provides a better understanding on construction of successful fluorescence vectors to facilitate future mixing population studies, but also deepens the understanding of the possible mechanism of transcriptional activity and plasmid recombination in *Giardia*.

Chapter 5

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Appendix I

Stable Plasmid pRFPneo Vector Sequences

Legend: Purple font is *Acc65*I site

Red font is *Hind*III site

Green font is glutamate dehydrogenase promoter

Blue font is *Cla*I

Red fluorescent protein is highlighted in red

Neomycin gene is highlighted in blue

5’TAGTTATTACTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTAAGCTGACCACA
AATAACGCCTTTAATTACAGGCGCCCCAGATTTTAAAATGCCTGGGTACCGCGGGCCCG
GGATCCACCGGTCGCCACCATGAGCGAGCTGATCAAGGAGAACATGCACATGAAGCTGT
ACATGGAGGGCACCGTGAACAACCACCACTTCAAGTGCACATCCGAGGGCGAAGGCAAG
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CGCCTTCGACATCCTGGCTACCAGCTTCATGTACGGCAGCAAAGCCTTCATCAACCACA
CCCAGGGCATCCCCGACTTCTTTAAGCAGTCCTTCCCTGAGGGCTTCACATGGGAGAGA
ATCACCACATACGAAGACGGGGGCGTGCTGACCGCTACCCAGGACACCAGCTTCCAGAA
CGGCTGCATCATCTACAACGTCAAGATCAACGGGGTGAACTTCCCATCCAACGGCCCTG
TGATGCAGAAGAAAACACGCGGCTGGGAGGCCAACACCGAGATGCTGTACCCCGCTGAC
GGCGGCCTGAGAGGCCACAGCCAGATGGCCCTGAAGCTCGTGGGCGGGGGCTACCTGCA
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TCGAGCAGCACGAGATGGCTGTGGCCAAGTACTGCGACCTCCCTAGCAAACTGGGGCAC
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CAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTC
ATCAATGTATCTTAAGGCGTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAA
ATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTAT
AAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCC
ACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATG

Chapter 5: References

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TAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGC
GCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTA
AATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAAT
AT

pTurboRFP forward primer →

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GTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCA
TGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTA
ACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGC
AGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTG
GAGGCCTAGGCTTTTGCAAAGATCGATAAGCTGACCACAAATAACGCCTTTAATTACAG
GCGCCCCAGATTTTAAAATGCCTGATCGATCAAGAGACAGGATGAGGATCGTTTCGCAT

← promoter reverse primer

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Chapter 5: References

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GATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCC
TTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATC
CCCTGATTCTGTGGATAACCGTATTACCGCCATGCAT 3′

Appendix II

Stable Plasmid pGFPneo Vector Sequences

Legend: Purple font is *Acc65*I site

Red font is *Hind*III site

Green font is glutamate dehydrogenase promoter

Blue font is *Cla*I

Green fluorescent protein is highlighted in green

Neomycin gene is highlighted in blue

5'TAGTTATTACTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTAAGCTGACCACA
AATAACGCCTTTAATTACAGGCGCCCCAGATTTTAAAATGCCTGGGTACCGCGGGCCCG

eGFP forward primer →

GGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGC
CCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAG
GGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAA
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ACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAA
GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGAT
CACTCTCGGCATGGACGAGCTGTACAAGGATGCGGCCGCGACTCTAGATCATAATCAGC

← eGFP reverse primer

Chapter 5: References

CATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAA
CCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATG
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CGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCC
GCGCTTAATGCGCCGCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGG
AACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAAT

pTurboRFP forward primer

AACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTCCTGAGGCGGAAAGAACC
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AGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTC
CCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGC
CCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCAT
GGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATT
CCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGATCGATAAGCTG
ACCACAAATAACGCCTTTAATTACAGGCGCCCCAGATTTTAAAATGCCTGATCGATCAA

promoter reverse primer

GAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCC
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Chapter 5: References

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GCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCAT
GCAT 3′